



Development of a Multi-Stage Vaccine against Paratuberculosis in Cattle

Thakur, Aneesh

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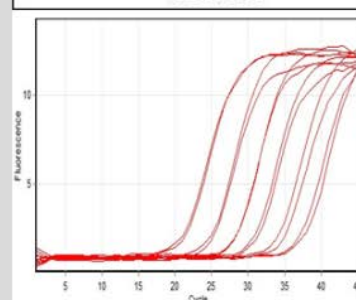
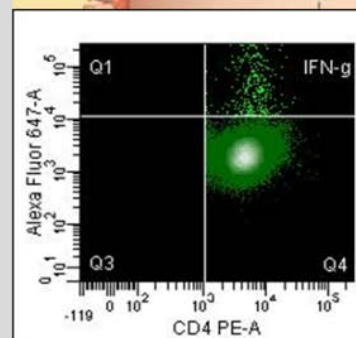
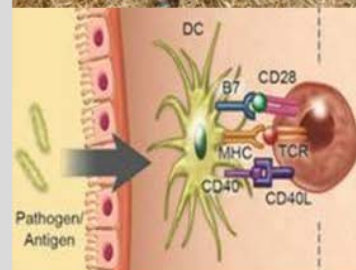
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Development of a Multi-Stage Vaccine against Paratuberculosis in Cattle

PhD Thesis

Aneesh Thakur

2012



Development of a Multi-Stage Vaccine against Paratuberculosis in Cattle

Aneesh Thakur

PhD Thesis

Section for Immunology and Vaccinology

National Veterinary Institute

Technical University of Denmark, Copenhagen

Copenhagen 2012

DTU Vet
National Veterinary Institute



Supervisors:

Professor Gregers Jungersen
Section for Immunology and Vaccinology
National Veterinary Institute
Technical University of Denmark

Senior Scientist Claus Aagaard
Infectious Disease Immunology
Vaccine Research and Development
Statens Serum Institut, Denmark

Assessment committee:

Senior Scientist Øystein Angen (Chairperson)
Section for Bacteriology, Pathology and Parasitology
National Veterinary Institute
Technical University of Denmark

Associate Professor Ad Koets
Department of Farm Animal Health
Faculty of Veterinary Medicine
Utrecht University, The Netherlands

Research Scientist Thomas Lindenstrøm
Vaccine Research and Development
Statens Serum Institut, Denmark

Front page illustration: Top to bottom: An experimental male Jersey calf (own picture); Uptake and presentation of antigen by dendritic cell to T cells (own picture); Intracellular IFN- γ release as measured through flow cytometry (own picture); Cycling SYBR Green quantitative PCR (own picture)

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PREFACE

The research work presented in this PhD thesis has been conducted entirely in the Section for Immunology and Vaccinology, National Veterinary Institute, Technical University of Denmark, Copenhagen in collaboration with Statens Serum Institut, Copenhagen from November 2009 to October 2012. The work was supported by a grant from the Danish Council for Independent Research, Technology and Production Sciences (FTP).

The thesis is structured as a review of paratuberculosis infection in cattle and following articles that are published or accepted or under revision in peer reviewed international journals:

Article 1:

Thakur, A., Aagaard, C., Stockmarr, A., Andersen, P., Jungersen, G. 2012. Cell-mediated and humoral immune responses after immunization of calves with recombinant multi-antigenic MAP subunit vaccine at different ages. Manuscript. Revised version accepted for publication in Clin. Vaccine Immunol.

Article 2:

Thakur, A., Riber, U., Davis, W.C., Jungersen, G. 2012. Increasing the *ex vivo* antigen-specific IFN- γ production in subpopulations of T cells by anti-CD28, anti-CD49d and recombinant IL-12 costimulation in cattle vaccinated with recombinant proteins from *Mycobacterium avium* subspecies *paratuberculosis*. Manuscript. Under revision in Vet. Immunol. Immunopathol.

Article 3:

Thakur, A., Pedersen, L.E., Jungersen, G. 2012. Immune markers and correlates of protection for vaccine-induced immune responses. Vaccine 30, 4907-4920.

Article 4:

Thakur, A., Aagaard, C., Riber, U., Andersen, P., Jungersen, G. 2012. A novel multi-stage subunit vaccine against paratuberculosis induces significant immunity and reduces bacterial burden in tissues.

Manuscript in preparation.

ABBREVIATIONS

ADCC	Antibody-dependent cell-mediated cytotoxicity
AFB	Acid-fast bacilli
AGID	Agar gel immunodiffusion
AMSC	Animal model standardization committee
APC	Antigen-presenting cell
ATP	Adenosine tri-phosphate
BSA	Bovine serum albumin
CAF	Cationic adjuvant formulation
CD	Cluster of differentiation
CFSE	Carboxyfluorescein succinimidyl ester
CFT	Complement fixation test
CFU	Colony forming unit
CMI	Cell-mediated immunity
CR	Complement receptor
CTL	Cytotoxic T-lymphocyte
CTLA	Cytotoxic T-lymphocyte antigen
CWD	Cell wall deficient
DCs	Dendritic cells
DDA	Dimethyldioctadecylammonium bromide
DIVA	Differentiating infected from vaccinated animals
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DTH	Delayed type hypersensitivity
ELISA	Enzyme-linked immunosorbent assay
Fc	Fragment crystallizable
FcR	Fc receptor
FCS	Foetal calf serum
Foxp3	Forkhead box p3
GALT	Gut associated lymphoid tissue
$\gamma\delta$ T cells	Gamma delta T cells
GC	Guanine cytosine
GM-CSF	Granulocyte macrophage colony-stimulating factor
HBSS	Hank's balanced salt solution
HE	Hematoxylin and eosin
Hsp	Heat shock protein
HTST	High temperature short time
ICS	Intracellular cytokine staining
IFN- γ	Interferon gamma
Ig	Immunoglobulin
IGRA	Interferon gamma release assay
IL-	Interleukin-
iMFI	Integrated mean fluorescence intensity
iNKT cells	Invariant NK T cells
IS	Insertion sequence
JDIP	John's disease integrated project
LAG	Lymphocyte activation gene
LAM	Lipoarabinomannan
LAMP	Lysosome-associated membrane protein

LJ	Lowenstein-Jensen
<i>M. avium</i>	<i>Mycobacterium avium</i> subsp. <i>avium</i>
<i>M. bovis</i>	<i>Mycobacterium bovis</i>
<i>M. tuberculosis</i>	<i>Mycobacterium tuberculosis</i>
MAC	<i>Mycobacterium avium</i> complex
MAIT	Mucosa-associated invariant T cells
ManLAM	Mannosylated lipoarabinomannan
MAP	<i>Mycobacterium avium</i> subsp. <i>paratuberculosis</i>
MAPK	Mitogen-activated protein kinase
MB	Middlebrook
M cell	Microfold cell
MHC	Major histocompatibility complex
mRNA	Messenger ribonucleic acid
MSV	Multi-stage vaccine
NFkB	Nuclear factor-kappa B
NK cells	Natural killer cells
NOD	Nucleotide-binding oligomerization domain
OD	Optical density
PAMPs	Pathogen associate molecular patterns
PBMCs	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
PE	Proline-glutamic acid
PP	Peyer's patches
PPD	Purified protein derivative
PPE	Proline-proline-glutamic acid
PRR	Pattern recognition receptors
qPCR	Quantitative real time PCR
rIL-12	Recombinant IL-12
RILP	Rab-7 interacting lysosomal protein
RNIs	Reactive nitrogen intermediates
RPMI	Rosewell park memorial institute
ROIs	Reactive oxygen intermediates
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SOD	Superoxide dismutase
TB	Tuberculosis
T _{CM}	Central memory T cell
TCR	T-cell receptor
T _{EM}	Effector memory T cell
T _{FH}	Follicular helper T cell
TGF-β	Transforming growth factor beta
T _H	T helper cell
T _M	Melting temperature
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor alpha
TRAF	TNF receptor-associated factor
T _{reg}	Regulatory T cell

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SUMMARY

Paratuberculosis is a chronic progressive granulomatous enteritis of ruminants caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP). Paratuberculosis in cattle is clinically characterized by weight loss, emaciation and diarrhea and subclinically by reduced milk production leading to considerable economic losses to farming community. Paratuberculosis is a staged infection in which young calves acquire the infection in the first months of life, may progress into a prolonged asymptomatic stage of about 2-5 years and may eventually become clinically infected animals. Vaccination with whole-cell live or inactivated vaccines prevents or delays the development of clinical stage of the disease but does not eliminate MAP and is usually accompanied by interference with bovine tuberculosis diagnostics as well as local tissue damage. Subunit vaccines with well-defined antigens in combination with a suitable adjuvant offer the possibility to avoid these limitations and induce a strong T helper 1 (T_H1) type immune response that has been associated with protection against MAP.

The aim of the study was to identify proteins from different stages of infection and formulate them into a multi-stage subunit vaccine with activation of protective immune response in experimentally challenged calves, with a focus on cell-mediated immune responses chiefly interferon gamma (IFN- γ) and polyfunctional T cells. The antigen composition of the vaccines was selected based on previous immunogenicity studies in cattle and experimental knowledge from *in vitro* and *in vivo* expression studies with *M. tuberculosis* proteins in mice (101). The vaccines were used to investigate the influence of age on vaccine-induced T cell responses and measuring vaccine-induced protective efficacy after experimental challenge. Effect of costimulation on vaccine-induced T cell responses and immune correlates of vaccine-induced protection were further characterized.

Early expressed and latency-associated MAP proteins were formulated in cationic adjuvant formulation (CAF) 01 and tested in calves through two different experiments, MAP multi-stage vaccine (MSV)-1 and

2. FET11 vaccine, a combination of a fusion protein of four early expressed MAP proteins and a latency-associated MAP protein formulated in CAF01 adjuvant was tested by an experimental MAP challenge in calves. FET11 vaccination at 16 weeks of age induced significant immune response and conferred protective immunity characterized by a mean $1.1 \log_{10}$ reduction in bacterial numbers in the gut tissues compared to control animals and was superior to a commercial whole-cell heat inactivated vaccine, Silirum® or FET11 vaccination at 2 weeks (Article 4). In both MSV experiments, most significant immune responses were observed against *Esx*-secretion system proteins and latency proteins (Article 1 and 4). However, the immunogenicity of two recombinant MAP proteins common in both studies was different, emphasizing the possibility of dynamics of MAP infection guiding the differential immune response. There was an association between age of vaccination and induced immune responses. Older animals (4 months) developed a more robust immune response (Article 1 and 4). Furthermore, no significant increase in the immune response was observed 8 weeks after second booster vaccination in MAP MSV-1 study (Article 1). However, decreased immune responses after one year period in MAP MSV-2 experiment, warrants the use of a booster vaccination. The experimental challenge of calves with mid-log-phase frozen stock MAP cultures correlated well with whole-blood IFN- γ responses to PPDj in advanced weeks of the study, which signifies PPDj response as a marker of MAP experimental infection (Article 4). This challenge study also supports the possibility of establishing a uniform and repeatable bovine MAP infection model involving large number of animals procured at different times. The results also show the potential application of quantitative real-time PCR (qPCR) for the evaluation of microbial load in tissues and vaccine efficacy (Article 4).

Costimulation of vaccine-induced *ex vivo* T cells significantly increased IFN- γ levels following use of anti-CD28 and anti-CD49d antibodies (Article 2). Recombinant interleukin IL-12 (rIL-12) also resulted in very high levels of IFN- γ production but was accompanied by high background levels. Thus enhanced antigen-specific immune response with anti-CD28/CD49d costimulation could be suitable for characterizing

vaccination or infection-mediated responses, while rIL-12 with a more T_H1 biased potentiation of antigen-specific IFN- γ production warrants its use for diagnostic purposes.

The results of this study also highlight the role of innate immune cells such as gamma delta ($\gamma\delta$) T cells and natural killer (NK) cells in paratuberculosis infection (Article 2). Antigen-specific IFN- γ production by $\gamma\delta$ T cells and NK cells was observed in vaccinated calves. Although the levels were low compared to CD4+ T cells, IFN- γ production by these innate effector cells might compensate the immature immune system of young calves to counteract MAP infection.

A number of immunological markers were discussed as potential vaccine-induced immune correlates with emphasis on infections requiring T cell-mediated immunity (Article3). Traditionally, neutralizing antibody titers are associated with vaccine-mediated immune protection. However, advancement of biological techniques has allowed identifying and appreciating immune markers as candidates for novel correlates of protection.

Taken together, this study has provided information on developing a multi-stage vaccine against paratuberculosis and has increased the knowledge regarding age of vaccination, experimental MAP infection, costimulation signals for measuring T cell responses, and immune correlates of protection.

RESUMÉ (DANISH SUMMARY)

Paratuberkulose er en kronisk fremadskridende granulomatøs enteritis hos drøvtyggere forårsaget af *Mycobacterium avium* subsp. *paratuberculosis* (MAP). Klinisk paratuberkulose hos kvæg er karakteriseret ved vægttab, afmagring og diarré og subklinisk paratuberkulose ved reduceret mælkeproduktion, hvilket medfører betydelige økonomiske tab for landbruget. Paratuberkulose er en gradvis infektion, hvor unge kalve smittes med infektionen i de første levemåned, som kan efterfølges af en langvarig asymptomatisk fase på omkring 2-5 år, og i sidste ende kan blive klinisk inficerede dyr. Vaccination med hele og levende celler eller inaktiverede vacciner forhindrer eller forsinker udvikling af den kliniske fase af sygdommen, men fjerner ikke MAP og medfører som regel en påvirkning af diagnostik for bovin tuberkulose samt lokale vævsskader. Subunit-vacciner med veldefinerede antigener i kombination med en egnet adjuvans giver mulighed for at undgå disse begrænsninger og inducerer et kraftig T-hjælper 1 (Th1) type immunrespons, som er blevet associeret med beskyttelse mod MAP.

Formålet med studiet var at identificere proteiner fra forskellige infektionsstadier og inkorporere dem i en flertrins subunit-vaccine til aktivering af beskyttende immunreaktion i eksperimentelt inficerede kalve, med fokus på cellemedierede immunresponses hovedsagelig interferon gamma (IFN- γ) og polyfunktionelle T-celler. Antigensammensætningen af vaccinerne blev valgt baseret på tidligere immunogenecitets-undersøgelser i kvæg og eksperimentel viden fra *in vitro* og *in vivo* ekspressionsstudier med *M. tuberculosis* proteiner i mus (101). Vaccinerne blev anvendt til at undersøge betydningen af alder på vaccine-inducerede T-cellereaktioner og til at måle den vaccine-inducerede beskyttende virkning efter eksperimentel infektion. Effekten af co-stimulering på vaccine-inducerede T-cellereaktioner og immun-korrelationer for vaccine-inducerede beskyttelse blev yderligere karakteriseret.

Tidlig udtrykt og latens-associerede MAP-proteiner blev formuleret i kationisk adjuvansformulering (CAF) 01 og testet i kalve gennem to forskellige eksperimenter, MAP flertrins vaccine (MSV) -1 og 2. FET11 vaccine, en kombination af et fusionsprotein af fire tidlig udtrykte MAP-proteiner og et latens-associeret MAP-protein formuleret i CAF01 adjuvant, blev testet ved en eksperimentel MAP re-infektion af kalve. FET11 vaccination ved 16 ugers alderen inducerede et signifikant immunrespons og gav beskyttende immunitet karakteriseret ved en middelværdi på 1,1 log₁₀ reduktion i antallet af bakterier i tarmvæv sammenlignet med kontroldyr, og var bedre end en kommerciel helcelle, varme-inaktiveret vaccine, Silirum® eller FET11 vaccination ved 2 uger (artikel 4). I begge MSV-eksperimenter blev de mest signifikante immunrespons observeret mod ESX-sekretionssystem-proteiner og latens-proteiner (artikel 1 og 4). Dog var immunogeniciteten af to rekombinante MAP-proteiner, som blev brugt i begge undersøgelser, forskellige, hvilket understreger muligheden for at dynamikken af en MAP-infektion kan inducere forskellige immunrespons. Der var en sammenhæng mellem vaccinationsalderen og inducerede immunrespons. Ældre dyr (4 måneder) udviklede et mere robust immunrespons (artikel 1 og 4). Desuden var der ikke signifikant stigning i immunrespons observeret 8 uger efter anden booster-vaccination i MAP MSV-1-studiet (artikel 1). Reducerede immunrespons efter et år i MAP MSV-2 eksperimentet, taler dog for anvendelsen af en booster-vaccination. Den eksperimentelle re-infektion af kalve med mid-log-fase, frosne stock MAP-kulturer korrelerede godt med fuldblods IFN- γ -reaktioner mod PPDj i de senere uger af studiet, hvilket understreger PPDj-reaktion som en markør for eksperimentel MAP-infektion (artikel 4). Dette infektions-studie understøtter også muligheden for at etablere en ensartet og reproducerbar bovin MAP-infektionsmodel med et stort antal dyr anskaffet på forskellige tidspunkter. Resultaterne viser også den potentielle anvendelse af kvantitativ real-time PCR (qPCR) til evaluering af mikrobielle belastning i væv og vaccineeffektivitet (artikel 4).

Co-stimulation af vaccine-inducerede *ex vivo* T-celler forøgede IFN- γ niveauerne signifikant efter brug af anti-CD28 og anti-CD49d antistoffer (artikel 2). Rekombinant IL-12 (rIL-12) resulterede også i meget høje

niveauer af IFN- γ produktion, men resulterede også i høje baggrundsniveauer. Derfor kan et forstærket, antigen-specifikt immunrespons med anti-CD28/CD49d co-stimulation være egnet til karakterisering af vaccinations- eller infektionsmedierede responser, mens rIL-12 med et mere Th1-rettet immunrespons begrænser anvendelsen til diagnostiske formål.

Resultaterne af denne undersøgelse fremhæver også rollen af medfødte immunceller såsom gamma delta ($\gamma\delta$) T-celler og naturlige dræberceller (NK) ved paratuberkulose-infektion. Antigen-specifik IFN- γ produktion af $\gamma\delta$ T-celler og NK-celler blev observeret i vaccinerede kalve. Selv om niveauet var lavt, sammenlignet med CD4+ T-celler, kan IFN- γ produktion af disse medfødte effektorceller kompensere for det umodne immunsystem hos unge kalve og modarbejde MAP-infektion.

En række immunologiske markører blev diskuteret som potentielle vaccine-inducerede immunkorrelater med vægt på infektioner, der kræver T-celle-medieret immunitet. Traditionelt er neutraliserende antistof-titrere blevet forbundet med vaccine-induceret immunbeskyttelse. Imidlertid har udviklingen af nye biologiske teknikker tilladt identifikation og anerkendelse af immune markører som nye korrelater for beskyttelse.

Samlet set har dette studie givet information omkring udvikling af en flertrins-vaccine mod paratuberkulose og har tilført viden om alder ved vaccination, eksperimentel MAP-infektion, co-stimulations-signaler til måling af T-celle responser, og immun-korrelater for beskyttelse.

INTRODUCTION

Paratuberculosis in Cattle

Paratuberculosis or Johne's disease is a chronic, progressive, and ultimately fatal enteric disease of mainly ruminants caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP). Paratuberculosis has a broad host range and can be found in many different species of wildlife and domestic ruminants. Paratuberculosis has an increasing economic impact worldwide and some zoonotic relevance due to increased association of MAP with Crohn's disease in humans (119). Paratuberculosis was first described in 1895 by Johne and Frothingham as a chronic enteritis in cattle (62). 11 years later in 1906, Bang differentiated the disease from tuberculosis (6). While the isolation of etiological bacterium was not achieved until several years later when Twort and Ingram cultured the bacterium and designated it as *Mycobacterium enteritidis chronicae pseudotuberculosis bovis*, Johne (168).

***Mycobacterium avium* subsp. *paratuberculosis* (MAP)**

MAP belongs to *Mycobacterium avium* complex (MAC) under the genus *Mycobacterium*, a group of acid-fast bacteria. MAC contains 28 serovars of two species: *Mycobacterium avium* and *Mycobacterium intracellulare*. *Mycobacterium avium* belongs to genus *Mycobacterium*, family *Mycobacteriaceae*, suborder *Corynebacterineae*, and order *Actinomycetales*. MAC has been divided into three subspecies: *Mycobacterium avium* subsp. *avium*, MAP, and *Mycobacterium avium* subsp. *silvaticum*. Among the three subspecies, only MAP is pathogenic in healthy hosts, while *Mycobacterium avium* subsp. *avium* and *Mycobacterium avium* subsp. *silvaticum* are unable to cause disease in healthy hosts but may do in immunocompromised hosts.

MAP is an intracellular pathogen. MAP is a small 0.5 x 1.5 µm, straight or curved rod-shaped, gram stain positive, acid-fast, obligate aerobic, non-motile bacterium that grows in clumps of 10-100 bacterial cells. MAP is usually considered a non-spore forming bacterium, however, a spore-like morphotype in

chronically starved MAP cultures have been found recently (85). MAP is characterized by very slow growth rate, with a generation time of approximately 30 hrs (84) and requiring 8-12 weeks for visible colony formation. MAP forms small 1-2 mm, usually white, domed, rough, non-pigmented colonies on artificial media, such as Herrold egg yolk or Lowenstein-Jensen (LJ) medium. MAP also has a dependence on siderophore mycobactin J, an iron-chelating cell wall component, for growth in primary cultures (165). The complete genome of the reference MAP strain K-10 (an isolate from Wisconsin dairy herd, 1990 and since then maintained in the lab; lab passage status is unknown) has been sequenced and is characterized by a single circular chromosome of 4,829,781 base pairs, relatively high guanine-cytosine (GC)-content (69%), and an abundance of insertion sequences (IS) and putative virulence factors, PE/PPE proteins (89). MAP genome sequence has annotated upto 4587 total genes. A total of 58 repetitive DNA sequences, insertion sequences (IS) have been recognized within the MAP genome, including 17 copies of *IS900*, 7 copies of *IS1311* and 3 copies of *ISMav2* (89). *IS900* is the first insertion sequence to be characterized in mycobacteria and is unique to MAP, though other mycobacteria have been reported to have *IS900* like sequences (38).

Like other mycobacteria, MAP possesses a thick cell wall containing 60% lipid and consisting of many layers. This multilayered cell wall confers on it the properties of acid-fastness, hydrophobicity (128), increased resistance to chemicals e.g. chlorine (182), and physical processes such as high-temperature short-time (HTST) pasteurization (48). The thick cell wall comprising of peptidoglycans, polysaccharides and lipids gives an advantage for the prolonged survival of MAP inside the host but leads to slow growth rate because of restricted nutrient uptake through the cell wall (36). A prominent lipopolysaccharide molecule of the cell envelope, lipoarabinomannan (LAM) plays a role in the pathogenesis of paratuberculosis (160). Broadly, MAP strains can be divided into two major groups or strain types, sheep (S) or Type I and cattle (C) or Type II. Application of molecular typing techniques revealed new strain types, intermediate (21) or Type III (32) and Bison type (189). However, recent data from whole-genome

sequencing supports only Types I and II with intermediate and bison strains part of Type I and Type II strains, respectively (2). Other characteristic features of MAP are the ability to persist for extended periods of dormancy in the environment (190) and water sources (188), biofilm formation (23), and aerosolization (188). In addition, cell wall deficient (CWD) forms of MAP have been reported in patients with sarcoidosis and Crohn's disease (57).

Epidemiology and the disease

Paratuberculosis is a disease of ruminants with cattle, sheep and goats regarded as the principle affected animals. However, paratuberculosis has also been diagnosed in a wide range of other free-ranging and domesticated ruminants such as bison, moose, riverine buffalo, bighorn sheep, deer, antelope, yak and camelids including camels, llamas and alpacas (96). MAP has been isolated from non-ruminant wildlife including rabbit, hare, fox, stoat, wild boar, badger, opossum, kangaroo, crow, wood mice and ferret (97). MAP has also been reported from horses, pigs, chicken, fish, and non-human primates (59).

The primary route of transmission of infection is through ingestion of milk, colostrum, feed or water contaminated with MAP microorganisms (51). Moreover, cows with clinical symptoms can transmit the infection vertically through the utero-placental route (192). Faecal shedding has been found to contribute significantly not only to cow-to-calf but also calf-to-calf transmission (4). A less common transmission route is through the semen of infected bulls (5). Spread of infection across herds is through introduction of infected animals to the healthy stock, with lateral transmission between adjoining pastures also possible (191). Young animals (< 12 months of age) are regarded most susceptible to paratuberculosis (86) but adult cattle can become infected too. Experimental infection studies in cattle have shown that between 4 months and 1 years of age, it is difficult to infect the calves and by 1 year, susceptibility of calves is similar to adult cattle. One speculation for the increased susceptibility of young

calves is the 'open gut' during the first 24 hours of birth, whereby MAP also cross the intestinal barrier along with colostral antibodies through pinocytosis. Age resistance in cattle has also been associated with the abatement of peyer's patches (PP) in the small intestine, considered as the portal of entry for MAP after birth and development of adaptive immune system in adult cattle. In addition, genetic variation in susceptibility for paratuberculosis has been described in cattle (77) and has been linked to single nucleotide polymorphisms in multiple genes like toll- like receptor (TLR)-2 (76) and nucleotide oligomerization domain (NOD)-2 (134) that are associated with MAP resistance or susceptibility. Furthermore, the magnitude of the MAP dose ingested will also affect the course of the infection. Animals that ingest a higher MAP dose tend to progress quickly to clinical stage of disease compared to ones ingesting lower dose.

Since the first recognition of paratuberculosis in dairy cattle, MAP has dispersed across species and geographical boundaries. This spread has coincided with industrialization, degree of economic consideration in animal agriculture and export of dairy heifers with supposedly prior MAP infection from Europe and North America to other continents of the world. Current prevalence estimates suggest that >50% of cattle herds in Europe (112) and >68% in North America are infected (91). Though, Norway (54) and Sweden (157) claim to be free of bovine paratuberculosis, positive cases from dairy cattle have been reported from both the countries. These herd level prevalence studies were carried out using an enzyme-linked immunosorbent assay (ELISA), and true prevalence could be even higher because the sensitivities of ELISAs were overestimated.

Pathogenesis

Paratuberculosis disease process starts with oral ingestion and passage of MAP through the epithelial barrier of the intestine, translocation through the mucosal epithelium by dome microfold (M) cells, phagocytosis in the intestinal mucosa and gut-associated lymphoid organs (GALT), and persistence in

subepithelial macrophages. This is followed by spread of infection to adjacent lymph nodes and dissemination. Macrophages are the vehicles for the dissemination of MAP through infected sites. It has been shown that MAP infected macrophages reach the mesenteric lymph nodes after crossing intestinal barrier within 1 h of surgical inoculation of intestine (198). Macrophages provide a favorable environment for MAP replication and persistence. In later stages, infected epithelial cells are sloughed into the intestine and passed with the faecal discharge. The number of MAP bacilli excreted in the faeces of clinically infected animals could exceed 10^8 cells per g faeces (18).

Paratuberculosis in cattle is characterized by a staged infection that can be divided into four stages according to disease severity, immune response and ability of diagnosis (Figure 1): stage 1, silent preclinical infection; stage 2, subclinical infection; stage 3, clinical infection; and stage 4, advanced clinical infection. Animals genetically resistant to paratuberculosis contain the infection and remain in the stage 1 without ever changing stages or only under conditions of an immunocompromise. In silent preclinical infection, susceptible animals remain asymptomatic carriers for the initial 2-4 years after infection with very little MAP shedding in the faeces, although cell-mediated immune (CMI) response and delayed type hypersensitivity (DTH) reaction may be detected. Intermittent MAP shedding in faeces detectable by faecal culture, no clinical signs, strong CMI responses and poor antibody responses characterizes stage 2. Stage 3 and 4 animals develop protein-losing enteropathy characterized by intermittent or persistent diarrhea. The hallmark clinical sign in cattle is watery or 'pipe-stream' diarrhea often with 'foam or bubbles' due to the high protein content in faeces. The intestinal wall thickens, caused by hypoproteinemia and edema due to decreased intravascular osmotic pressure (20). This results in malabsorption of nutrients, gradual weight loss despite normal or increased food intake, submandibular oedema, reduced milk production by 20% or more, and decreased fertility (70). Bacterial shedding is persistent with strong production of antibodies against MAP and a waning CMI response. The infection can disseminate to several extra-intestinal sites, including lymph nodes. Animals in

advanced clinical disease become weak, emaciated, and suffer from profuse diarrhea. Intermandibular oedema or bottle jaw is characteristic of this stage and animals eventually die of dehydration and cachexia (166). However, most animals are culled before reaching this terminal stage. It has been suggested that for every clinically confirmed paratuberculosis infected cow there are another 15-25 infected cows in a herd (184). This phenomenon has been described as the 'iceberg effect.'

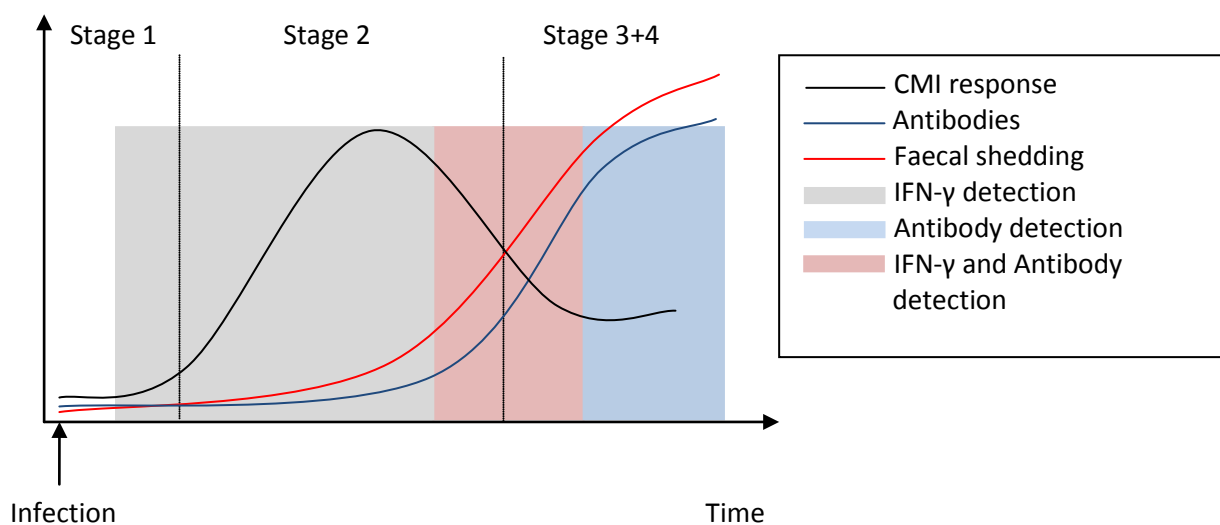


Figure 1: Stages of MAP infection in cattle with dogma accompanying diagnostic options (Figure Aneesh Thakur).

Disease pathology

Macroscopic changes associated with paratuberculosis in cattle include intestinal wall thickening, lymphadenitis leading to lymphangiectasis, and lymph node enlargement (Figure 2). Affection of the mucosal epithelium of the distal ileum is the major component of the pathogenesis and is characterized by an oedematous wall with raised corrugations (Figure 2). The serosal and mesenteric lymphatic vessels are dilated and thickened. The mesenteric lymph nodes are pale, swollen and oedematous. Extraintestinal lesions in liver, hepatic lymph nodes and kidney have been reported (53). Interestingly, the degree of intestinal lesions often doesn't correlate with the clinical outcome (13).

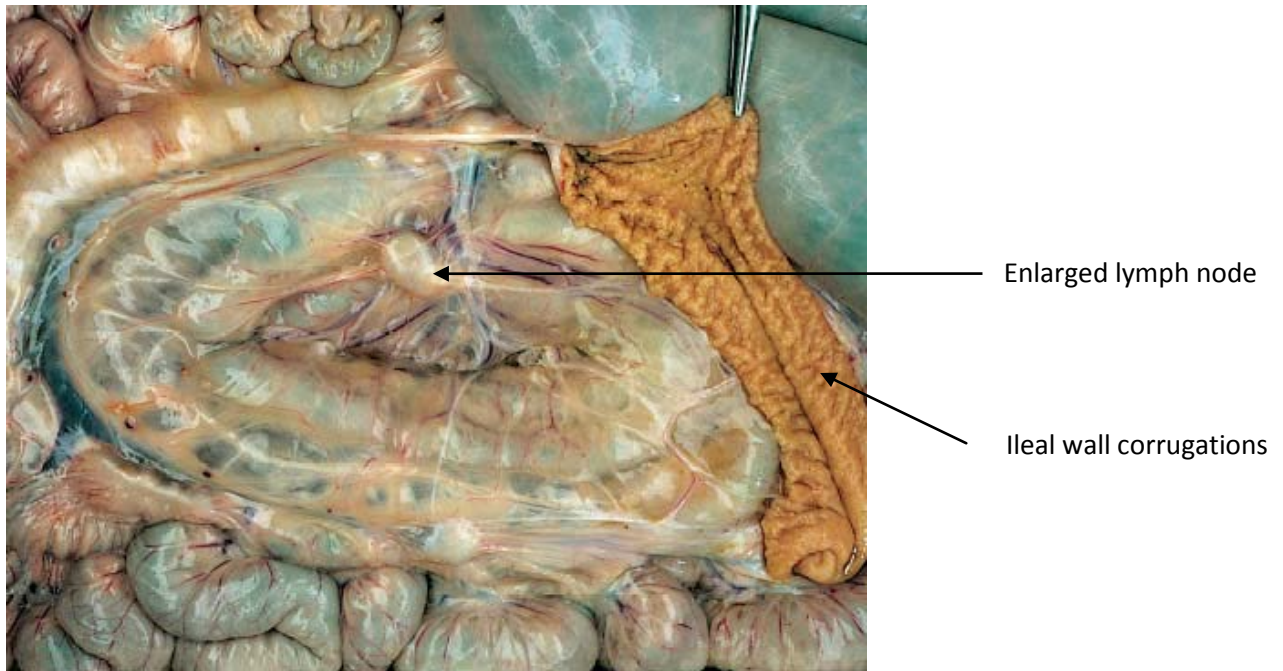


Figure 2: Ileal wall thickening and corrugations and lymph node enlargement (Photo Johne's disease: Animal Health Australia www.animalhealthaustralia.com.au).

Histopathological lesions are characterized by focal, multifocal or diffuse granulomatous inflammation of the intestine and mesenteric lymph nodes. In both human and bovine tuberculosis, chronic granulomatous lesions are typical and are characterized by a central caseation, surrounded by macrophages, epithelioid cells and multinucleate giant cells which in turn are covered by lymphocytes, macrophages and a fibrous capsule. However, there is never a tuberculous granuloma formation in bovine paratuberculosis although multiple granulomas are seen in goats (92) and caseous lesions are observed in deer (95). Lesions in bovine paratuberculosis have been classified into mild, moderate or severe (13). Mild lesions are characterized by the presence of giant cells and few macrophages in the intestinal villi and paracortical zone of mesenteric lymph nodes with no acid-fast bacilli (AFB). In moderate form, there are many macrophages and giant cells extending up to intestinal mucosa and submucosa and mesenteric lymph nodes and few AFB. Severe form has profuse infiltration of macrophages and giant cells in all layers of intestine and lumen of lymphatic vessel, blunted villi and

distended intestinal crypts. Cellular infiltration extends to paracortical as well as subcapsular region of mesenteric lymph node and AFB are demonstrable in large numbers. Depending upon the extent of severity, lesions can also be classified as focal, multifocal or diffuse. Focal lesions extend up to intestinal villi or ileocaecal and jejunal lymph nodes. Multifocal lesions spread till the lamina propria of ileum, ileal and jejunal lymph nodes. Diffuse lesions further can be paucibacillary, multibacillary or intermediate type (46). Diffuse paucibacillary type is characterized by profuse lymphocytic infiltration and few AFB, while multibacillary lesions have a large numbers of macrophages with abundant AFB and few lymphocytes. Paucibacillary form is generally accompanied by higher CMI response while multibacillary form is often characterized by strong antibody response. Intermediate form shows features of both paucibacillary as well multibacillary types with AFB corresponding to macrophage presence and may thus represent the transitional nature of the intermediate form.

Diagnosis

Diagnosis of bovine paratuberculosis can be divided into assays that detect either the infectious agent or a MAP-specific immune response in the host. The diagnostic assays that detect MAP in the clinical samples such as faeces or tissues could be culture or polymerase chain reaction (PCR) based methods. These methods have high specificity (98-100%) but low sensitivity (about 70%) in animals with clinical disease. The sensitivity is even poor (about 25%) for subclinically infected animals (114, 185). Although pathology induced by MAP is characteristic, diagnosis based on pathological changes, demonstration of AFB or immunohistochemisry and *in situ* hybridization conducted on tissue sections is not specific (186). Since MAP is an obligate pathogen, cultivation and identification of MAP is a definitive diagnostic test. However, actual challenge is the identification of subclinically infected animals, which intermittently shed low number of bacteria in the faeces and are usually negative on standard antibody tests (114).

Culturing

Cultivation of MAP is a highly specific method and requires some critical steps such as decontamination of clinical samples, enrichment, prolonged incubation periods, and identification using genotyping means. Both solid as well as liquid media can be used to culture MAP such as LJ medium, Harrold's egg yolk medium, Middlebrook (MB) 7H10 or 11 agar, and MB7H9 broth. Incubation periods vary from 12-20 weeks on solid medium and 8-12 weeks on the liquid medium. Enrichment of the media using mycobactin J is essential for primary culturing of MAP. Egg yolk, sodium pyruvate, dyes such as brilliant green, methylene blue and antimicrobials such as malachite green, vancomycin, penicillin G, amphotericin B have been used to stimulate the growth of MAP. Radiometric detection of C¹⁴-labelled carbon dioxide produced in mycobacterial cultures was adapted for MAP using BACTEC12B liquid medium and detection through BACTEC 460 system (29). A new fluorescent detection system called BACTEC MGIT 960 based on identification of a fluorescent signal generated in the liquid medium as the oxygen is consumed is gaining acceptance for detection of MAP (158). Culturing of MAP is a costly and time-consuming procedure. Culture of samples pooled from more than one animal or the environment, is usually practiced for determining the herd prevalence. In addition to faecal samples, tissues such as intestine and associated lymph nodes, milk, blood and environmental samples, including soil, water and pasture have been used for the isolation of MAP.

PCR

Confirmation of the MAP cultures through IS900 PCR is a highly sensitive (100%) method. However, direct PCR on the clinical samples have been found to be of low sensitivity due to PCR inhibitors that can lead to false-negative results. Thus standard PCR based assays don't give a practical alternative to other diagnostic methods. To counter this problem, new methods have been developed such as immunomagnetic separation (47) and hybridization capture (98). Despite a lot of work in the field, the most common target of conventional PCR is still the IS900 gene. A comparison of 13 PCR assays found

IS900 PCR assay to be most sensitive with an assay detection limit of 0.1 pg DNA (172). Few other markers have proved useful for the identification of MAP including F57, ISMAP02, ISMav2, heat shock protein (Hsp) X, gene 251 and 255. Nested PCR is another sensitive method to detect MAP but suffer from risk of cross-contamination. Multiplex assays incorporating more than one marker at a time such as IS900, F57 and 16S PCR assays have been developed to confirm MAP (164). qPCR is yet another sensitive technique that allows 'absolute' quantification of MAP in clinical samples. Further, it can be combined with reverse transcriptase PCR for MAP mRNA detection and expression analysis (9).

Immunoassays

MAP diagnosis can also be performed with immunoassays that depend on the interaction of host with the microorganism, leading to a measurable immune response. These assays can detect either the CMI (tuberculin skin sensitivity or IFN- γ production) or humoral responses (IgG1 response). Immune responses in paratuberculosis were believed to be characterized into earlier pro-inflammatory reaction dominated by IFN- γ and IgG2 and later anti-inflammatory reaction dominated by IgG1 antibodies (110). However, recent knowledge has challenged the existence of T_H1 over T_H2 dormancy in MAP (10). In fact, both responses may occur simultaneously and thus pose a challenge for the accurate diagnosis. In addition, the disease progression, faecal shedding and antibody responses don't follow a set order (93). Few infected cows produce antibodies several years prior to continuous shedding of MAP, while in others, antibodies may not be detectable in early stages of infection.

CMI based assays

There are two CMI based diagnostic assays for the detection of MAP infection: intradermal tuberculin skin test and IFN- γ release assays (IGRAs). Tuberculin skin test measures the delayed type hypersensitivity after intradermal inoculation of johnin or avian purified protein derivative (PPD). However, skin testing suffers from the drawback of cross-reaction with environmental bacteria and MAP

or *M. bovis* vaccines, in addition to more handling of animals. Skin testing has been replaced to a large extent with the IFN- γ assay, first described for the diagnosis of bovine tuberculosis in 1990 measuring secreted IFN- γ in response to an antigen (196).

In IGRAs, whole-blood samples are cultured with MAP antigens or PPD antigen and IFN- γ release in 24 hrs cultures is measured through ELISA. These assays have the advantages of shorter timeframe, repeatability, and objective interpretation of results and less animal handling. But suffer from low specificity, complex logistics and higher price. Current IGRAs use PPD as the stimulating antigen. PPD is a poorly defined mixture of mycobacterial antigens from different strains and has lower specificity due to cross-reactive antigens. Further, PPD used for IGRAs is not standardized because the genomic variability is very high among the organisms used for preparing PPD (143). Thus well-defined specific antigens should be used for improving the performance of IGRAs. One of the other limitations for the use of IGRAs in their current form is the variable sensitivity (0.13 to 0.85) and specificity level (0.88 to 0.94) in cattle (114). Further, non-specific IFN- γ production in calves less than 15 months of age has been observed (117). Another requirement for the IGRAs is the immediate processing of whole-blood samples within 8 hrs of transport to the lab to maintain the specificity. However, this issue has been addressed by the use of costimulatory cytokines such as IL-7, IL-12, and IL-18. In spite of all the limitations, IGRAs are still promising in early detection of paratuberculosis (56, 63). A negative association between IGRAs results obtained before first calving and milk antibody ELISA results at different ages after calving has been observed, indicating the key role of CMI responses in the control of paratuberculosis (102).

Serology based assays

MAP-specific antibodies in the serum samples can be detected by assays such as complement fixation test (CFT), agar gel immunodiffusion test (AGID) or ELISA. Of all three, ELISA is the most sensitive test with age related increase in the sensitivity (113). Lower specificity of the ELISA tests due to cross-

reacting antibodies have been improved by the absorbing sera with *Mycobacterium phlei*. Specificities ranging from 98.8% (28) and 99.7% (129) have been obtained using this method. Consequently, the sensitivity of the assays is compromised in animals without clinical signs as compared to the higher sensitivity in clinically affected animals (114). Thus one of the limitations of the ELISA tests is poor sensitivity in early stage infected animals posing a challenge for the timely diagnosis of the infection.

Several commercial ELISA kits for bovine paratuberculosis are currently available, and many studies have compared their accuracy. Similar to CMI based assays, ELISA tests also face the challenge of having suitable antigen representing the entire range of immunodominant antigens for MAP. Most widely used antigen is PPA-3, which is the *M. avium* strain 18 protoplasmic antigen. While other used antigens include whole-cell sonicated extract, PPD, and protoplasmic antigens, and cell wall antigen lipoarabinomannan (LAM) but they show variable potency and cross-reaction. Another important consideration for serological diagnosis of MAP infection is the antibody isotype. As there is predominance of IgG1 and IgG2 isotypes in different stages of MAP infection (78), therefore, it is important to measure the right antibody isotype responses.

Milk antibody ELISA is also used for diagnosis of MAP infection and offers the advantages of individual and bulk milk sampling, ease of sampling, low cost of testing, and herd surveillance. A positive ELISA is useful for predicting that an animal would subsequently become infectious. Cattle with repeated positive milk ELISA are found to be more likely to be MAP shedders in near future compared to other with fluctuating immune responses (109). However, many factors have to be taken into consideration for the interpretation of milk ELISA results including stage of lactation, milk yield and days in milk. Stage of lactation has a major effect on the odds of testing positive in milk antibody ELISA, particularly during the first days of lactation, where the odds are 3-27 times higher than mid-lactation (111). In the advanced stages of paratuberculosis, a state of anergy may occur that leads to almost no milk antibody

responses (16). It was observed that in herd surveillance programs, days in milk 1-5 should be excluded, while milk yield data for the effect of dilution should be taken into account in order to predict test results corresponding to disease progression (111).

Thus for the accurate diagnosis of paratuberculosis, the performance of both the CMI and serological based assays ought to be improved by incorporating MAP-specific antigens. New methods such as spot protein array have been developed for initial antigen screening (8). A number of characterized MAP antigen candidates with diagnostic potential have been summarized and categorized into secreted, cell wall and membrane, lipoprotein, heat shock and hypothetical proteins (100). Using a cocktail of novel immunogenic antigens of MAP is a good approach to increase the sensitivity and specificity of the ELISA test for diagnosis of paratuberculosis (104). Despite all the improvements the fundamental question remains the same i.e. what is the purpose of the applied diagnostic test? Our research group has earlier addressed this question and highlighted the significant impact of the diagnostic target condition as well as the purpose of testing on the utility of available tests (64).

Paratuberculosis: Immunobiology

Uptake and Innate immune response to MAP

After oral ingestion of MAP, the bacilli enter the mucosal surface of intestinal tissue through peyer's patches (PP). PPs are lymphoid aggregates within GALT and allow selective transport of antigens.

Microfold (M) cells are the portal of entry for MAP inside PP. M cells, unlike enterocytes, are characterized by lack of brush border epithelium, digestive enzymes and surface mucus and thus provide an unobstructive path for ingested bacilli (40). MAP has fibronectin attachment protein in the cell wall that is activated during its passage through acidic contents of abomasum, which allows opsonization through fibronectin protein in the body fluids. Fibronectin then serves as a bridge to bind MAP with fibronectin receptor $\alpha 5\beta 1$, on the luminal surface of M cells (141). In addition, pattern

recognition receptors (PRR) such as TLR4 and platelet activation factor receptor have been involved in the bacterial uptake through M cells (169). Recent evidence also suggests MAP uptake by enterocytes (139). After crossing the intestinal epithelial layer, MAP is expelled at the basolateral side and taken up by the subepithelial macrophages or dendritic cells (DCs). Various PRR have been implicated in the entry of mycobacteria into macrophage or DCs such as TLRs, NACHT-like receptors including NOD1 and NOD2 receptors, mannose receptor, complement receptors (CR1, CR3, and CR4), immunoglobulin receptor (FcR), scavenger receptors (e.g. CD163), inhibitory receptors (such as C-type 2 lectins like DC-SIGN/CD209), and others (124). TLRs, TLR2 and TLR4 are important in the detection and initiation of adaptive immune response by activation of intracellular signaling pathways through MyD88, mitogen-activated protein kinase (MAPK), and nuclear factor- κ B (NF- κ B), leading to induction of cytokines (61). TLR2, TLR4 and NOD2 receptors have been reported to be involved in MAP recognition by macrophages (41), highlighting the key role of TLRs in mediation of innate immune responses. TLR-mediated intracellular signaling leads to production of cytokines and chemokines such as IL-1, IL-6, IL-8, IL-12 and tumor necrosis factor alpha (TNF- α) that initiate proinflammatory immune responses (187).

Once inside the macrophages, MAP persists by surviving the microbicidal properties of macrophages by inhibiting the maturation of phagosomes and preventing apoptosis. Lower expression of transferrin, an early phagosome marker and higher expression of lysosome-associated membrane protein-1 (LAMP-1), a late phagosome marker have been shown on phagosomes with live MAP (55). Interference with the normal course of phagosome maturation into a phagolysosome has been linked to an inhibition of phagosome acidification, which in turn leads to poor microbicidal functions such as nitric oxide, reactive oxygen species and lysosome hydrolases (82). Markers from Rab GTPase family, Rab5 and Rab7 that are critical for intracellular signaling for phagosome-lysosome fusion have been found to be altered by mycobacterial infection (19). A recent study has shown reduced phagosome-lysosome fusion in MAP infected human monocyte cell lines evidenced by reduced recruitment of Rab-7 interacting lysosomal

protein (RILP) in Rab-7 staining phagosomes containing live MAP (71). In addition, MAPK pathway was found to promote MAP survival by preventing acidification of phagosomes (147). Pathogenic MAP also evades apoptosis of macrophages and there is evidence for the longer survival of MAP using this strategy (195).

NK cells are innate effector cells that have been shown to restrict the growth of mycobacteria through the production of IFN- γ and cytolytic activity (33). NK cells are usually found in a resting state and need activation signals for effector functions provided by DC (15). In addition, NK cells with memory functions have been described (161). Thus NK cells serve an important link between innate and adaptive immune system (Figure 3). In MAP infection, NK cells produce IFN- γ (117), but a larger role still remains to be elucidated.

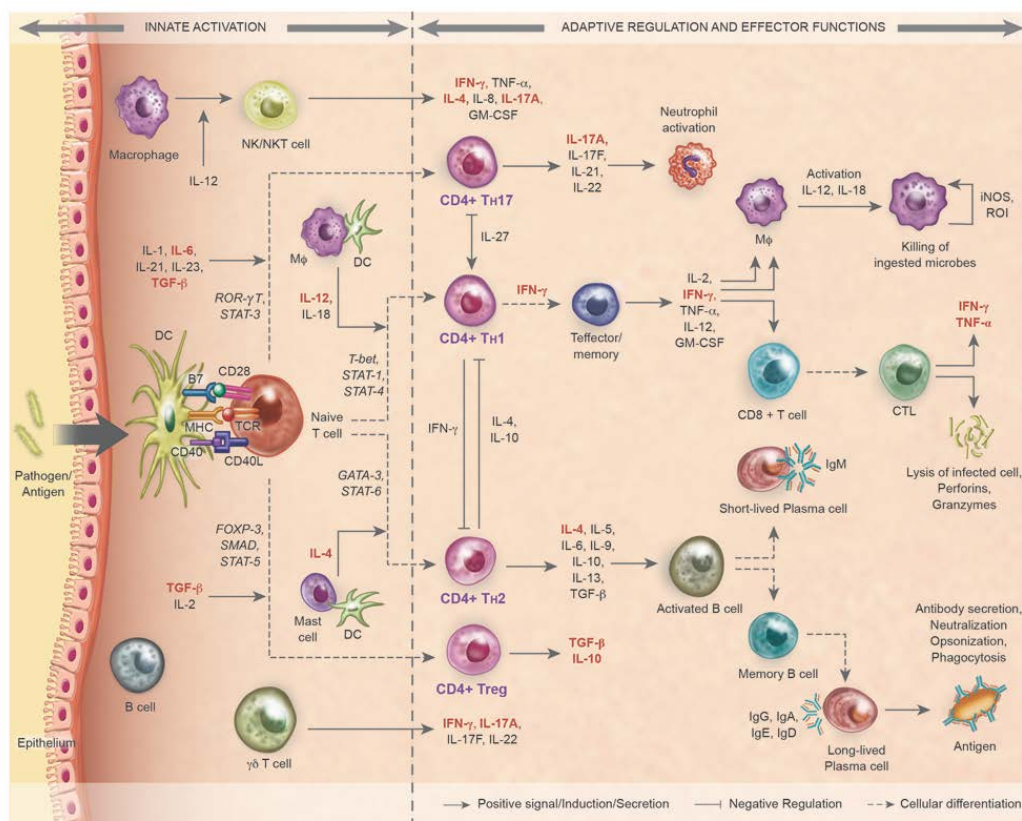


Figure 3: Illustration of induction and regulation of CMI and humoral immune responses (Figure Aneesh Thakur).

Cell-mediated immune response to MAP

IFN- γ is regarded as a canonical cytokine in mycobacterial infections including paratuberculosis (149).

The chief producers of IFN- γ include innate immune cells such as NK, invariant NK T (iNKT) cell and $\gamma\delta$ T cells and adaptive immune cells, CD4⁺ and CD8⁺ T cells. IFN- γ has the ability to synergize or antagonize the effects of cytokines, growth factors, and signaling pathways and is important for the activation of macrophages, which in turn exerts increased phagocytosis and bactericidal effect. MAP may remain intact or replicate in persistently infected macrophages, or are degraded for antigen presentation to T cells. Activated macrophages in turn produce increased amounts of IL-1 α , TNF- α , IL-6, IL-8, IL-12, IL-18, and IL-23. Enhanced production of IL-1 α leads to upregulation of an antiapoptotic protein, TNF receptor-associated factor 1 (TRAF1) that in turn limits signaling of CD40, TNF- α , and CD95 (FAS) and programmed cell death (25). The resultant effect is the recruitment and prolonged survival of macrophages at the MAP infection site leading to diffuse granulomatous inflammation. IL-8, a chemotactic factor recruits other immune cells such as neutrophils and lymphocytes to the affected area (106). IL-12, IL-18 and IL-23, produced by activated macrophages stimulate IFN- γ production by CD4⁺ T cells and $\gamma\delta$ T cells (120). Once activated, macrophages or dendritic cells emigrate the site of MAP encounter to PP or the mesenteric lymph nodes, the sites of T cell activation and critical signaling events. Several adhesion molecules such as CD62L, CD44, VLA-4 and chemokine receptors such as CCR7 and its ligands, CCL19 and CCL21 are associated with activated macrophage or dendritic cell migration to the lymph nodes (136). The cascade of cytokines then leads to activation of T cells in combination with antigen presentation. T cell activation is an instructively programmed process and is divided into 3 key signals (50)(Figure 4). During Signal 1, macrophages or DCs present antigen associated with major histocompatibility complex (MHC) class I to CD8⁺ or class II molecules to CD4⁺ T cell, respectively. Signal 2, the costimulatory cell surface signal involves interaction of CD80 (B7-1) and CD86 (B7-2) on the antigen presenting cells (APCs) with the CD28 on T cells. While, Signal 3 is provided by cytokine cascade described above. Activated T cells will in turn travel through peripheral blood and home to mucosal

MAP affected sites in order to exert their effector functions (Figure 3). Activated T cells produce IL-2, which helps in the clonal expansion of CD4⁺ helper T cell and CD8⁺ cytotoxic T cells. T cells produce a myriad of cytokines and based upon their effector functions can be differentiated into distinct T cell lineages.

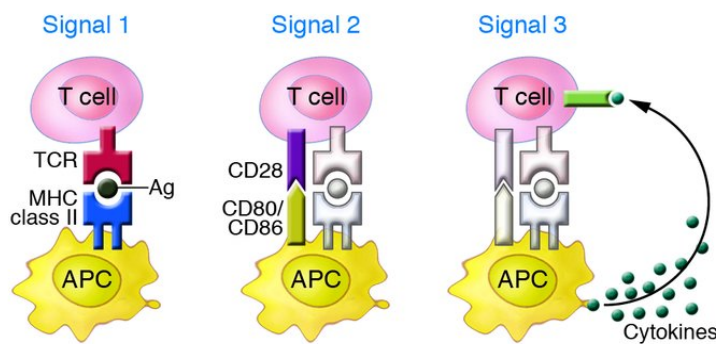


Figure 4: Signals for T cell activation. From Gutcher and Becher, 2007 (50).

T cell subsets

CD4⁺ helper T cells (T_H) are predominant proliferating cell populations and major producers of IFN- γ in MAP infection (149). T_H cells can differentiate into several types of effector cells: T_H1, T_H2, T_H17, and T_H3/regulatory T cells, each characterized by production of distinct sets of cytokines. T_H1 cells produce IFN- γ , TNF- α and IL-2, activate macrophages and generate CD8⁺ cytolytic T lymphocytes (CTL), all required for control of MAP. The signature T_H1 cytokine, IFN- γ , enhances IgG2a class-switching (25). T_H2 cells produce IL-4, IL-5, and IL-13 that enhance antibody production and antibody-dependent cellular responses against MAP. The signature T_H2 cytokine, IL-4, instructs B cells to produce antibodies. In paratuberculosis, early and late stages of disease have been associated with a predominance of T_H1 and T_H2 cells, respectively. However, recent findings suggests towards a less distinct predominance of both T cell subsets in mycobacterial infections including paratuberculosis (10, 148). T_H17 cells produce IL-17, IL-21 and other cytokines/chemokines, recruit neutrophils and have been shown to play a possible role in the pathology of MAP infection in animals (130, 145) as well as humans (119) (Figure 3). T_H3/regulatory

T (T_{reg}) cells serve to maintain self and peripheral tolerance. T_{reg} cells mediate suppression of effector cell by producing IL-10, transforming growth factor beta (TGF- β) and IL-35 (Figure 3). Yet, new T cell subtypes have been identified recently including follicular helper T cells (T_{FH}), T_H9 cells, T_H22 cells, mucosa-associated invariant T cells (MAIT), and alternative MHC 1b restricted T cells with possibilities of identifying even more subsets in the future (116). The T_H1 and T_H2 immune profile may represent the most stable of all cell lineages and being programmable to memory phenotype (162), however, other cell types are more plastic both *in vitro* and *in vivo* with T_{FH} appearing to be the most fluid subset. New findings argue that although some cytokines are selectively produced, many are broadly expressed and that helper cells can change their phenotype and therefore, their plasticity and heterogeneity should not be ignored (116) (Figure 5).

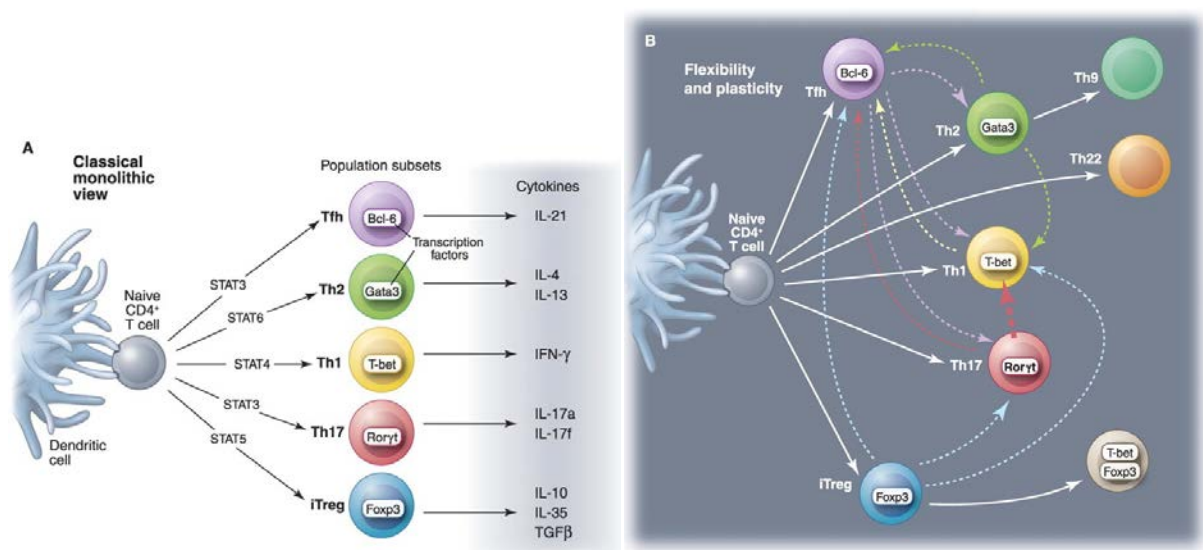


Figure 5: Helper T cell differentiation (a) The classical T cell lineage model (b) Flexibility and plasticity of helper T cells. From O'Shea and Paul, 2010 (116).

Cytotoxic CD8⁺ T cells (CTL), are another major fraction of $\alpha\beta$ T cells that also play a role in defense against MAP by removing infected cells through contact-dependent lysis and release of cytokines (24). Activation of CD8⁺ T cells through presentation of mycobacterial antigen from phagosome on the MHC class I molecule occurs through the process of cross-presentation. CTL activates apoptotic cell death in

the mycobacteria infected cell releasing preformed secretory granules including perforin, granzymes, and granulysin or FAS/FAS ligand pathway (197). CD8+ T cells also contribute to IFN- γ production in MAP infected animals (152). Although CD4+ T cells have been found to play a role in the early stages of paratuberculosis, CD8+ T cells may play a more significant role in late infection (81).

$\gamma\delta$ T cells, a distinct lineage of T cells expressing $\gamma\delta$ T cell receptor (TCR) have increasingly been identified to play a role in host defense against mycobacterial infections as well as serving a bridge between innate and adaptive immunity (Figure 3). $\gamma\delta$ T cell number can exceed 50% of the circulating T lymphocytes in blood and lymph in the young animals (193). $\gamma\delta$ T cells are characterized by absence of MHC restriction, enormous $\gamma\delta$ TCR diversity and their ability to recognize soluble proteins and non-protein molecules such as mycobacterial lipids by CD1 molecule. In addition, bovine $\gamma\delta$ T cells respond directly to pathogen associated molecular patterns (PAMPs) and may even act as APCs (22). $\gamma\delta$ T cells produce cytokines and have cytotoxic properties and play a yet undefined role in early MAP infection. Quantities of IFN- γ secreted by $\gamma\delta$ T cells in response to MAP antigens appear to be lower than produced by CD4+ T cells, but the response is antigen-specific (144). $\gamma\delta$ T cells were not able to restrict the growth of MAP after challenge but were involved in the formation of epithelioid granuloma instead (163). However, in another study, loss of CD4+ T cell in the final stages of paratuberculosis have been associated with an increase in $\gamma\delta$ T cells (75).

Regulatory T (T_{reg}) cells play a critical role in the maintenance of tolerance by regulating immune responses to self and foreign antigens and also in mycobacterial infections. T_{reg} cells are a very heterogeneous T cell population with distinct subsets including thymus derived, naturally occurring CD4+CD25+FoxP3+ T_{reg} and many subsets of peripherally developed, induced or adaptive T_{regs} (CD4+CD25-) such as IL-10 secreting T_R1 cells and TGF- β secreting T_H3 cells. T_{reg} cells suppress the activation, proliferation and effector functions of CD4+, CD8+, NK, B cells and APCs through cytokine-

mediated suppression by IL-10, TGF- β , IL-35, CD25 or by direct cell-to-cell contact-mediated suppression through CTL antigen (CTLA) 4, lymphocyte activation gene 3 (LAG-3), granzyme A and B, and FAS-FAS ligand (135). In mycobacterial infections with long subclinical phase including paratuberculosis, reduced effector T cell activity has been associated with the development of T_{reg} cells. In paratuberculosis clinically infected cattle, MAP antigen stimulated peripheral blood mononuclear cells (PBMCs) secrete IL-10 (27), while the infected tissues secrete elevated levels of TGF- β (72). In animals infected with paratuberculosis, it was proposed that T_{reg} cells could play a role in the advanced stages of infection by antagonizing T_H1 response (31). IL-10 produced from PBMCs of cattle with subclinical paratuberculosis was found to reduce IFN- γ secretion (31). IL-10 production from PBMCs of MAP infected animals have been shown to be predominantly derived from monocytes (92, 94, 159). Recently, CD4+CD25+T_{reg} cells producing IL-10 have been identified in bovine paratuberculosis (26). However, $\gamma\delta$ T cells have also been associated with regulatory activity in MAP infection in cattle (17).

Humoral immune response to MAP

Antibody responses generally correlate to mycobacterial-elicited pathology in accordance with the belief that *Mycobacteria spp.* induce antibody primarily late in the course of infection. Little data is available that strongly associates B cell responses with paratuberculosis, although an antibody response is usually associated with the onset of clinical paratuberculosis. Antibodies can play a role in the extracellular phase of MAP when translocating from lumen to intestinal macrophages or from dying infected macrophages to young uninfected monocytes and macrophages. Antibodies may even facilitate uptake of MAP on their target through macrophage Fc receptors (FcR) and it could even be speculated that increased levels of antibodies are mediators rather than indicators of disease progression. Two subsets of B cells have been recognized based on the expression of surface marker CD5, CD5+ B cells or B1 cells and CD5- B cells or conventional B cells. Once activated, B cells can differentiate into either antibody secreting plasma cells or memory cells. Antibodies mediate their protective effects through a wide panel

of classical effector functions such as neutralization, opsonization, complement fixation, oxidative burst, and antibody- dependent cell-mediated cytotoxicity (ADCC) (Figure 3). In addition to antibody production, B cells can act as APCs and play a role in the activation of CD4⁺ T_H2 cells. Studies in mycobacterial infections have linked B cell function with disease state such as delayed dissemination of *M. tuberculosis* from lungs to spleen and liver in B cell knockout mice (11). In clinically MAP infected cattle, a high increase in B cell numbers was reported compared to subclinical cattle (177). Likewise, a decrease in the CD5dim B cell population along with a concomitant increase in CD5bright B cells (equivalent to B1a cells) was observed in subclinically infected cattle, reflecting a shift in host immunity during the disease process (151). In MAP experimental challenge studies in calves, a strong B cell response was observed with a difference between routes of challenge (150). MAP-specific antibody responses were found to be higher in calves challenged intraperitoneally than the calves challenged orally. Although MAP antibody responses are usually observed in clinical stages, they can be measured from 2 years of age with the current diagnostic assays. However, a transient antibody response was detected 70 days post-infection against defined recombinant MAP antigens (7). MAP infection is usually accompanied by a shift in the percentage of B cells in the peripheral blood, with high numbers in clinical infected cattle compared to subclinically infected or control cattle (153).

In cattle, a distinction between T_H1 and T_H2 associated antibody isotypes have been described with IgG2 and IgM isotypes classified as type 1, while IgG1 and IgA classified as type 2 isotypes (39). In paratuberculosis, shifting of the two isotypes has usually been attributed to a loss of type 1 reactivity. However, loss of type 2 reactivity have been described to few MAP antigens such as Hsp70 and LAM (78). All these findings suggest a role for B cells in regulating pathogenesis of MAP infection and an optimism to develop recombinant antigens from different stages of disease to fully understand the role of B cells in paratuberculosis.

Immune memory

Memory T cell pool is heterogeneous and comprised of cells with different migratory and effector capacities (136). Thus some memory cells express chemokines and immune markers required for migration to lymph nodes and are called central memory T cells (T_{CM}). Other memory T cells do not express lymph node homing markers but migrate to tissues and infection sites to execute effector functions and are referred to as effector memory T cells (T_{EM}). Both these memory T cell types have increasingly been associated with strong immune responses in secondary infections and have potential as immune correlates of vaccine-induced protection (Figure 6).

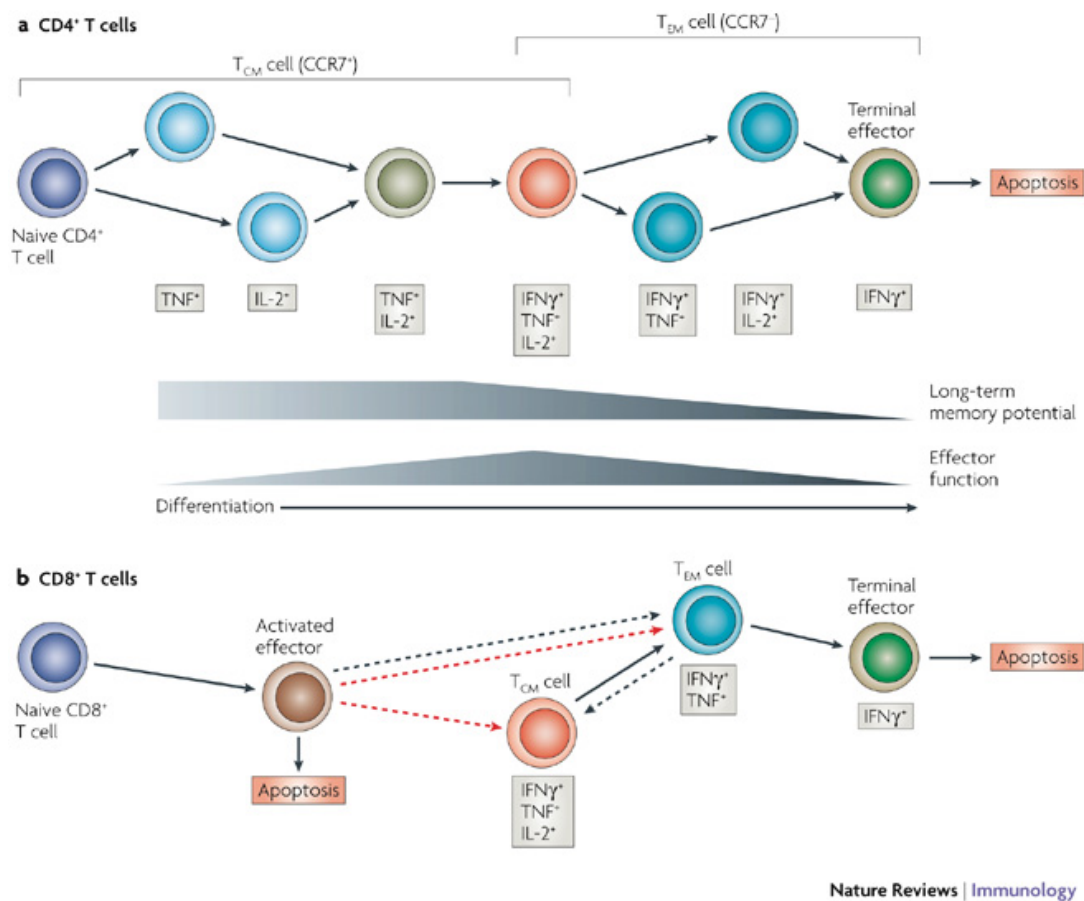


Figure 6: Models for effector and memory T cell differentiation. From Seder et al., 2008 (142).

Unlike human and mice memory T cells, the T_{CM} and T_{EM} cells are not yet fully characterized in ruminants. One of the reasons is the lack of species-specific antibodies and markers for labeling central and effector memory T cells. In experimental MAP infection studies in calves, an increased expression of activation markers such as CD25, CD26 and memory marker CD45RO⁺ have been observed among CD4⁺ and CD8⁺ T cells following restimulation of PBMCs with MAP antigens (3, 81, 175). L- Selectin or CD62L, mediates specific adhesion to peripheral lymph node vascular addressins targeting resting lymphocytes to areas of high antigen concentration with lymph nodes. Low expression of CD62L (CD2⁺CD62L⁻) has been used to define memory T cells in paratuberculosis (181). CD44 is a T cell marker that is up regulated on lymphocytes upon activation and has been associated with memory T cells with effector functions. In *M. bovis* infection in cattle, expression of CD25 and CD44 was found to be increased in proliferating T cells in the periphery compared to decreased expression of CD62L (176). This over expression was accompanied by a high IFN- γ release confirming their effector memory status. In ruminants, T_{CM} have not been fully characterized as yet due to lack of specific antibodies against chemokine receptors such as CCR7. However, T_{CM} and T_{EM} have been described in cattle following *Mycoplasma mycoides* subsp. *mycoides* infection based on CD62L and CD45RO memory markers along with gene expression of CCR7 (167).

MAP persistent infection

One of the hallmarks of mycobacterial infections is their ability to maintain infection in the host even in the presence of inflammation, innate immune defense and a robust adaptive immune response, and thereby giving rise to persistent infections, which can be life-long at times. MAP is an intracellular bacterium and uses several survival tactics to adopt a persistent life style including modulation of initial uptake, immunoescape and immunomodulation. Different routes of entry can alter the intracellular fate of MAP e.g. complement receptor (CR1) and mannose receptor mediated uptake of MAP doesn't stimulate the production of superoxide anion (60) and NADPH oxidase (156), respectively. Also, CR3-

mediated uptake doesn't affect the intracellular fate of MAP. MAP cell wall lipoglycan, mannosylated LAM (ManLAM) induces marked expression of both IL-10 and TNF- α through TLR-2 signaling. High IL-10 expression in turn suppresses both the inflammatory and immune response to MAP.

MAP can escape the potential microbicidal mechanisms of phagocytes by inhibiting phagosome-lysosome fusion and phagosomal acidification (195). This has been recognized as a major immunoescape mechanism in persistent MAP infection. MAP infected bovine macrophages have the ability to produce superoxide dismutase (SOD) and evade killing by reactive oxygen intermediates (ROIs) including superoxide anion, hydrogen peroxide, and hydroxyl radical produced during the process of phagocytosis (179). Likewise, MAP infected macrophages produce insufficient amounts of reactive nitrogen intermediates (RNIs) such as nitric oxide (199).

Apoptosis of mycobacteria infected macrophages is another mechanism for the intracellular killing of bacilli. Mycobacteria including MAP resist apoptosis for their prolonged survival (195). Recently, it was reported that MAP-infected macrophages show a drastically low ability to activate caspases and subsequently suppresses host cell apoptosis (65). Adenosine tri-phosphate (ATP) released from resting cells as well as cells undergoing apoptosis or necrosis can kill MAP through the activation of purinergic receptors. Elimination of extracellular ATP has been found to increase the survival of MAP-infected bovine monocytes (194).

MAP favors its survival by exploiting ongoing granulomatous inflammatory reaction by recruiting new macrophages that are used for bacterial dissemination. MAP then interferes with macrophage activation and production of cytokines including IL-8, IL-12 and TNF- α (179) or promotes rapid IL-10 production. In addition, MAP also attenuates MHC class I and II expression in bovine monocyte-derived macrophages

(180). Thus MAP employs a broad anti-immune strategy to survive and escape host immune elimination that leads to a chronic persistent infection.

Immune markers and correlates of protection

In infections with long periods of latency such as mycobacterial infections, measuring the immune response from clinical endpoints is difficult and thus the development of vaccines will rely on demonstration of immunological correlates of protection. A correlate of protection is a specific immune response to a vaccine that is closely related to protection against infection or disease or other defined endpoint (125). However, for persistent infections with long latency such as mycobacterial infections, it is difficult to define the absolute correlates of protection. CMI responses, chiefly IFN- γ production has served as the basis for the selection of candidate vaccine antigens in mycobacterial infections. However, a few recent studies have challenged the role of IFN- γ in protection against tuberculosis infection (42, 66). At the same time, in paratuberculosis, instead of a T_H1 CMI response, a strong antibody response has been observed following Hsp70/DDA vaccination (74). The role of antibodies in mycobacterial infections has already been appreciated by others (45). T cell quality i.e. T cells capable of producing IFN- γ , TNF- α and IL-2 cytokines, referred to as 'polyfunctional T cells' have been demonstrated as a hallmark of vaccine-induced protective immunity as well as infection in several chronic intracellular infections (30, 142). However, other studies have failed to correlate risk of disease or protection with polyfunctional T cells (42). Polyfunctional T cells have been demonstrated recently in cattle naturally infected with *M. bovis* (183). A number of other immunological parameters have been studied as markers of protection in mycobacterial infections but have yielded inconsistent results (174).

Control: whole-cell vaccines

After the paratuberculosis disease recognition in 1895 and isolation of the causative bacteria in 1911, Vallee and Rinjard soon introduced vaccination for the control of paratuberculosis in the year 1934

(171). This vaccine consisted of a live non-virulent MAP strain with an adjuvant made of olive oil, liquid paraffin and pumice powder. The results of this vaccine prompted the use of vaccine against paratuberculosis in other countries. In the 20th century, a number of vaccines were developed and were available commercially such as killed, live attenuated or improved whole-cell based vaccines. Most of the currently used vaccines use same basic formulation of whole bacilli in an oil adjuvant. Vaccination in cattle does not play a central role in paratuberculosis control in any country. All of these vaccines prevent the clinical signs and reduce faecal shedding but were unable to prevent the onset of infection (170). An important disadvantage of these vaccines is the interference with serological diagnosis of paratuberculosis and tuberculosis infections as these vaccines does not hold potential for a DIVA (differentiating infected from vaccinated) (80, 155). Thus vaccination against MAP might not allow disease eradication, yet interfere with national tuberculosis eradication programs and poses a challenge for the export of cattle. There are also safety concerns with inactivated vaccines as localized tissue damage has been reported in humans by accidental self-inoculation (123).

MAP vaccines: recent development

New paratuberculosis vaccines have been developed over the years with emphasis on subunit vaccines, mostly MAP recombinant proteins with adjuvants (67, 74), DNA vaccines (132, 140), expression library immunization (58), and mutant MAP strains (14, 121). Many immunodominant protein antigens inducing strong T_H1-type immune response have been developed and tested in MAP experimental infections in calves but found to have low efficacies: members of antigen 85 complex, Ag85A, Ag85B, and Ag85C (107); Hsp65 (GroEL) and Hsp70 (DnaK) (79); lipoprotein P22, a Lppx/LprAFG family of mycobacterial lipoprotein (37); PPE family proteins, MAP1518 and MAP3184 (108); SOD (MAP2121c) (144); and MPP14, a 14-kDa secreted protein (118). A cocktail of recombinant proteins (Ag85A, Ag85B, Ag85C and SOD) in combination with MPL or MPL+IL-12 adjuvant were tested in experimental MAP infected calves but failed to induce complete protection (67). Subunit vaccines have the advantage that

well-characterized antigens can be used to induce immune responses. Moreover, it has been shown that candidate Hsp70/DDA subunit vaccine did not interfere with the specificity of comparative skin test, is suitable for developing antibody based assays and it serves as a DIVA vaccine (137, 138) but it does not give complete protection. The development of new vaccines against paratuberculosis needs to consider several aspects including safety, efficacy, absence of interference with tuberculosis and paratuberculosis diagnosis, possibility for a DIVA strategy, and ability to control onset and progression of infection in animals. Many experimental MAP challenge studies in cattle have highlighted the importance of parameters such as experimental challenge conditions, dose, inoculation route, challenge strain, age, samples, analysis and experimental endpoints to be critical for the establishment of a bovine infection model.

Experimental MAP infections

Different MAP strains of cattle were found to produce different immunological profiles in experimentally infected calves (154). In terms of age, older animals were found to be more resistant to infection (103). Infectious dose ranging from 1×10^6 colony forming units (CFU) to $4-8 \times 10^{10}$ CFU have been used in different experimental challenge studies (52). In addition to the common oral challenge, other routes such as intratonsillar, subcutaneous, intravenous, intrauterine, intranasal, and transtracheal routes have been used in cattle. Variable infection levels have been reported using either oral, subcutaneous or intravenous routes of inoculations (87). Similarly, variation in genetic susceptibility between breeds and blood lines has been observed in cattle (77). International guidelines for the experimental challenge models for paratuberculosis have been proposed by the Johne's Disease Integrated Project (JDIP) Animal Model Standardization Committee (AMSC) in year 2007 (52). In addition to ruminants, mouse models have been used for the testing of vaccine candidates. Although mice are not the target species for paratuberculosis, they serve as a valuable tool for the preclinical testing of potential vaccine candidates owing to availability of a broader immunological toolbox, various genetic

backgrounds of inbred mice and the low maintenance cost (52). One of the other important needs for the development of new vaccines against paratuberculosis is the evaluation of vaccine efficacy and potency. Due to slow progression of disease, lengthy experimental trials, and associated high costs, it is imperative to define vaccine immune correlates and protection markers to develop potency tests.

AIMS OF THE STUDY

The main aim of the thesis was to identify antigens from different phases of MAP infection and formulate them into a vaccine with targeted activation of protective CMI responses. Development of a multi-stage vaccine against paratuberculosis has the potential to significantly increase the health and welfare of infected cattle and reduce the environmental contamination with this supposedly zoonotic bacterium.

Hypotheses

1. The protein expression profile of MAP is different in acute versus chronic stages of the infection
2. A vaccine stimulating CMI against both acute and latent stage antigens will be able to decrease bacterial load in newly infected animals and prevent the re-activation of infection leading to clinical disease in older animals.
3. Immune response to MAP vaccine will be influenced by the age of vaccination.
4. Polyfunctional T cells will be an immunological correlate of vaccine-induced protection.

Research Strategy

The clinical course of paratuberculosis is usually described as a progressive infection, but although animals become infected in early life, the typical clinical case of paratuberculosis is an adult cow that has not shown any clinical (or immunological) sign of infection for several years. Rather than a simple straightforward progression of disease, we believe it may, therefore, be more correct to describe the slow pathogenesis of paratuberculosis as an initial acute, actively dividing phase followed by a latent, resting phase where the infection is hiding from adverse immune reactions inside macrophages and only replicates slowly, if at all. During the latent phase, the bacterium expresses a different profile of proteins, and at a low level, compared to the active phase. Thus in order for a modern vaccine to be efficiently protective against new infections and also fight existing latent infections, the vaccine must

address these different sets of proteins expressed in different phases of the infection. We call this vaccine strategy a 'multi-stage' vaccine. Such a vaccine should be able to decrease the bacterial load in newly and latently infected animals and prevent re-activation of dormant bacteria. Furthermore, the use of well-defined antigens in the vaccine makes possible the use of standard ELISA assays with complimentary antigens (e.g. a paratuberculosis LAM ELISA) to develop a DIVA vaccine. Using this strategy, two prototype vaccines Ag85b-ESAT6-Rv2660c and Ag85b-TB10.4-Rv2659c have been proven to be superior to Ag85b-ESAT-6 and Ag85b-TB10.4 (e.g. the vaccines without the therapeutic antigen), both as a preventive multi-stage vaccine and as a therapeutic vaccine against latent tuberculosis in animal models such as mice and non-human primates. We have followed a similar strategy and identified MAP antigens from the different phases, formulated corresponding fusion proteins in an adjuvant with specific targeting of cell-mediated immune responses, and tested this vaccine in an experimental MAP bovine model.

Specific Objectives

- To formulate a multi-stage vaccine against paratuberculosis with strong activation of cell-mediated immune responses based on antigens from MAP representative of both acute and latent stages of the infection
- To characterise the vaccine-induced immune responses and the short-term protective efficacy of the vaccine against an experimental MAP infection in young calves
- To provide the basis for a subsequent evaluation of long-term therapeutic effect of the vaccine in chronic infected cattle

These specific objectives were investigated through following studies:

1. Influence of age of vaccination on MAP vaccine-induced T cell responses
2. Costimulation and vaccine-induced *ex vivo* IFN- γ production by T cell subsets
3. Immune markers and correlates of protection for vaccine-induced immune responses

4. A multi-stage MAP vaccine-induced immune responses and protective efficacy after experimental challenge

METHODOLOGICAL CONSIDERATIONS

The work described in this thesis is based on two experimental studies in calves named MAP multi-stage vaccine (MSV)-1 and MAP MSV-2. In brief, MAP MSV-1 experiment was conducted to evaluate the effect of age on vaccine-induced immune response and the calves were vaccinated but not MAP challenged. In the MAP MSV-2 experiment, all the calves were challenged with MAP and then divided into different vaccine groups and a non-vaccine group.

Animals

Male jersey calves were used for the two experimental studies. All the calves were obtained at the mean age of 2 weeks from a farm, which by the Danish paratuberculosis surveillance program had a true prevalence equal or close to zero at all milk-antibody samplings from September 2006 to January 2011 (101). For the MAP MSV-1 experiment, 27 calves were used that were divided into three groups- Vac2w, Vac8w and Vac16w. On the other hand, 28 calves were used for the MAP MSV-2 experiment and were randomly distributed into four groups- Early FET11, Late FET11, Silirum® (CZ Veterinaria, Spain) and Control. Silirum® is a heat-inactivated vaccine containing 2.5 mg of the culture of strain 316F of MAP combined with an adjuvant consisting of highly refined mineral oil.

Inoculum preparation, challenge and vaccination

All the calves in the MAP MSV-2 experiment were challenged with live MAP bacilli at the age of two weeks. The strain of MAP used for the challenge of the calves was a Danish clinical isolate, Ejlskov 2007 isolated from the faeces of a 4 year old clinically MAP shedder cow in 2007. The MAP culture was growing on LJ medium slants. A single colony from the LJ slant was resuspended in MB7H9 medium supplemented with 10% oleic acid-albumin-dextrose complex plus 0.05% Tween 80 and 2% Mycobactin J. Culture was propagated in the medium as depicted through figure 7 and stocked with 15% glycerin at

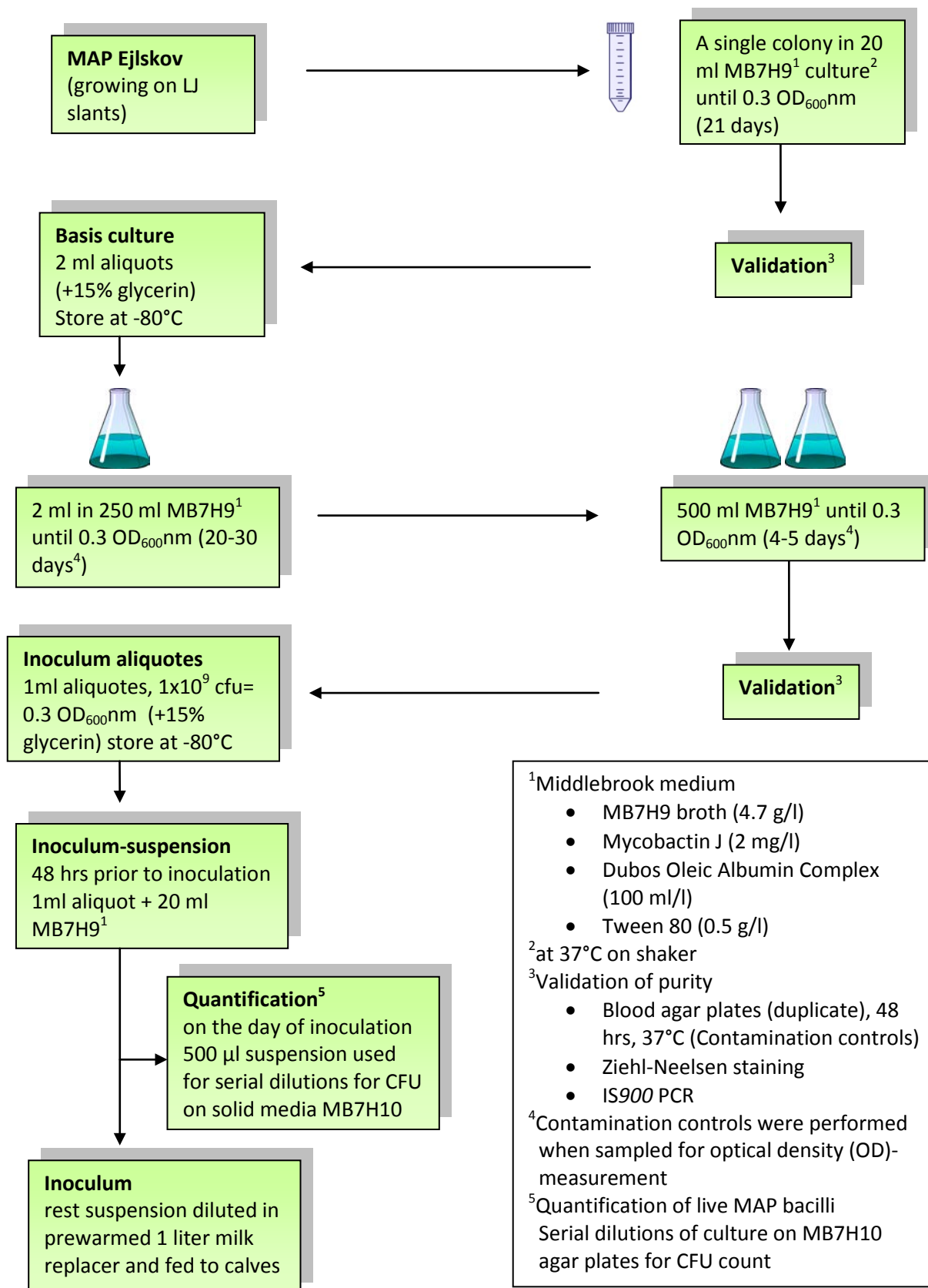


Figure 7: Preparation of MAP Inocula (Figure Aneesh Thakur).

-80°C at the concentration of 1×10^9 CFU/ml. The number of CFU was determined using the pelleted wet weight method that estimates approximately 1×10^7 CFU/mg pelleted wet weight (52). Two days before inoculation, 1 ml inocula aliquot was thawed in water bath (37°C) and added to pre-warmed media (MB7H9 with supplements; 20 ml) to prepare MAP inoculum for individual animal. The whole suspension was then incubated on a shaker at 37°C for 48 h. For inoculation, calves were fed 20 ml MAP suspension in a liter of pre-warmed (38°C) commercial milk replacer three times within a week period.

As the principal route of MAP infection in calves is through the contaminated milk at an early age, we believe that our method of experimental infection of calves is relatively close to the natural infection. Infection dose of 1×10^9 CFU/ml was selected based on published MAP experimental studies and considering the recommendations of the International committee for standardization of MAP challenge models (52). Vaccination of the calves in both studies was performed by inoculating MAP recombinant proteins formulated in CAF01 adjuvant sub-cutaneously in the right mid-neck region about 7 cm ahead of the prescapular lymph node. We believe that this method of vaccination should result in a better antigen presentation and strong T_H1 immune response based on prior knowledge using CAF01 adjuvant in mice models of human tuberculosis (90).

Antigens incorporated in multi-stage vaccine in both MAP MSV-1 and MAP MSV-2 experimental studies were selected based on previous immunogenicity studies in MAP infected and free cattle and experimental knowledge from *in vitro* and *in vivo* expression studies with *M. tuberculosis* proteins in mice (99, 101). Figure 8 illustrates the approximate molecular weights of the five vaccine proteins used in the MAP MSV-2 experimental study.

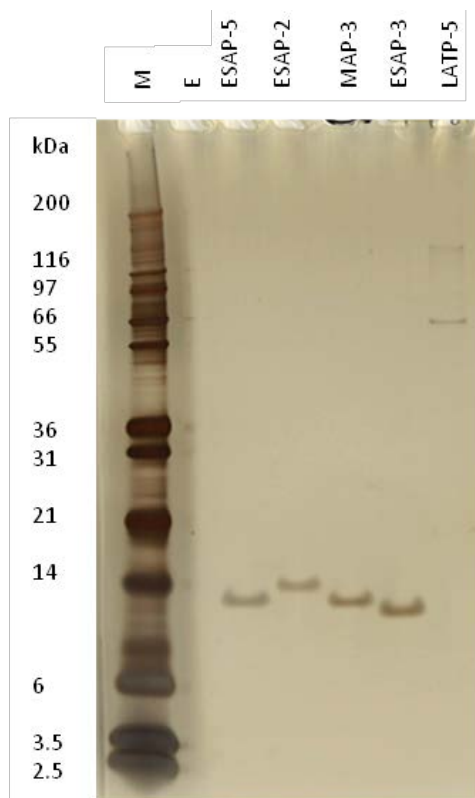


Figure 8: SDS-PAGE analysis of purified recombinant MAP proteins. Lane M: marker of protein size (kDa); Lane E: empty; following lanes show 1 μ g of each of ESAP-5, ESAP-2, MAP-3, ESAP-3, and LATP-5. Protein bands were visualized by silver staining (Photo Aneesh Thakur).

Whole-blood IFN- γ test

The antigen-specific IFN- γ secretion in 18-20 hrs whole-blood culture supernatants was determined by use of an in-house monoclonal sandwich ELISA as has been described previously (102). We also considered the incubation time of 48 and 96 hrs but found higher background values at both time points. Earlier we have reported enhanced IFN- γ responses in whole-blood cultures using recombinant IL-12 or anti-IL-10 antibodies (99). Therefore, we tested using recombinant bovine IL-12 and IL-18 (porcine and human) in the blood cultures for measuring IFN- γ responses. After recombinant IL-12 or IL-18 costimulation, a significant increase in the IFN- γ response was observed. The increase in the IFN- γ response using either porcine or human recombinant IL-18 was found to be identical. We did, however,

find that recombinant IL-12 or IL-18 as well as their combination apparently resulted in higher background values, at least in samples from few vaccinated animals as well as an adjuvant control animal in MAP MSV-1 (not reported) experiment. Incubation and costimulation kinetics were not pursued further in the whole-blood cultures for IFN- γ measurement.

Antigen-specific serum IgG1 ELISA

An in-house developed and optimized indirect ELISA was applied for the demonstration of serum IgG1 antibody responses in MAP MSV-1 experiment. In this study, serum IgG2 levels were also measured but were found to be very weak for any useful interpretation and thus were excluded from serological response analysis. The important considerations for quantitative and qualitative solid phase immunoassays including ELISA are choice of solid phase surface, coating proteins and their coating concentrations, blocking buffer, secondary antibody or conjugate, positive control and the substrate. For antigen coating, we tested MAP recombinant proteins at coating concentrations of 0.2 $\mu\text{g/ml}$, 1 $\mu\text{g/ml}$ and 5 $\mu\text{g/ml}$ and selected 0.2 and 1 $\mu\text{g/ml}$ as optimum for our setup. Bovine serum albumin (BSA) as well as casein was tried as blocking buffer. However, an increased background response was observed in plates using casein as blocking buffer in two adjuvant control animals in MAP MSV-1 experiment. So the BSA buffer was employed as blocking buffer in this assay. Anti-bovine IgG1: HRP conjugate was titrated and 1:500 dilution (final conc. 0.5 μg) was found to be optimum. As a positive control included on all plates, we used the serum samples of a vaccinated calf with consistently high antibody responses, while the sera sample on the day of vaccination from the same calf was used as negative control.

Multicolor Intracellular cytokine staining and flow cytometry

As we hypothesize earlier that polyfunctional T cells could be an immunological correlate of vaccine-induced protection, so we used multi-color flow cytometry to substantiate our statement. However, due

to non-availability of bovine specific IL-2 antibody at that point of time and some difficulties with bovine anti-TNF- α staining, we only managed to measure IFN- γ production by flow cytometry. With bovine TNF- α antibody we observed surface as well as intracellular expression of TNF- α as has been reported earlier in one study (49). We were not able to differentiate bovine TNF- α expression between PBS stimulated and SEB stimulated cultures in few animals. With the recent availability of bovine specific IL-2 antibody, polyfunctional T cells producing IFN- γ , TNF- α and IL-2 have been demonstrated in cattle naturally infected with bovine tuberculosis (183) and we were able to replicate the polyfunctional T cell staining protocol in our lab (Figure 9). In addition, we also looked at the expression of surface markers including CD25, CD44, CD62L and CCR7.

For the measurement of antigen-specific IFN- γ production, a protocol was developed in order to identify the specific cell populations producing IFN- γ in the stimulated PBMCs cultures including CD4+, CD8+, $\gamma\delta$ T cells and NKp46+ cells. Two different 18-20 hrs culture protocols were tried for measuring IFN- γ release, one protocol included Brefeldin and Monensin A for the last 6 hrs and the other used Brefeldin and Monensin A for the last 12-14 hrs. The first protocol was used due to high production levels of IFN- γ in PBMCs cultures. For the surface phenotyping of IFN- γ producing cells, anti-CD2, anti-CD3, anti-CD4, anti-CD8, anti-WC1, and anti-NKp46 antibodies were used. In six of the MAP MSV-1 experimental animals, the anti-CD4 monoclonal antibody (Clone CC8) didn't stain at all and anti-CD4 antibody (Clone IL-A11) was, therefore used for the rest of the study. Potentiation of the IFN- γ responses was also studied using recombinant IL-12, porcine or human recombinant IL-18, anti-CD28, anti-CD49d, or anti-CD5 antibodies. We also looked into CD4+, CD8+ and $\gamma\delta$ T lymphocytes proliferation and activation using carboxyfluorescein succinimidyl ester (CFSE) proliferation assay and expression of activation markers CD25, CD44, and CD62L in 5 days old cultured PBMCs. A high expression of activation markers was observed among proliferating lymphocytes.

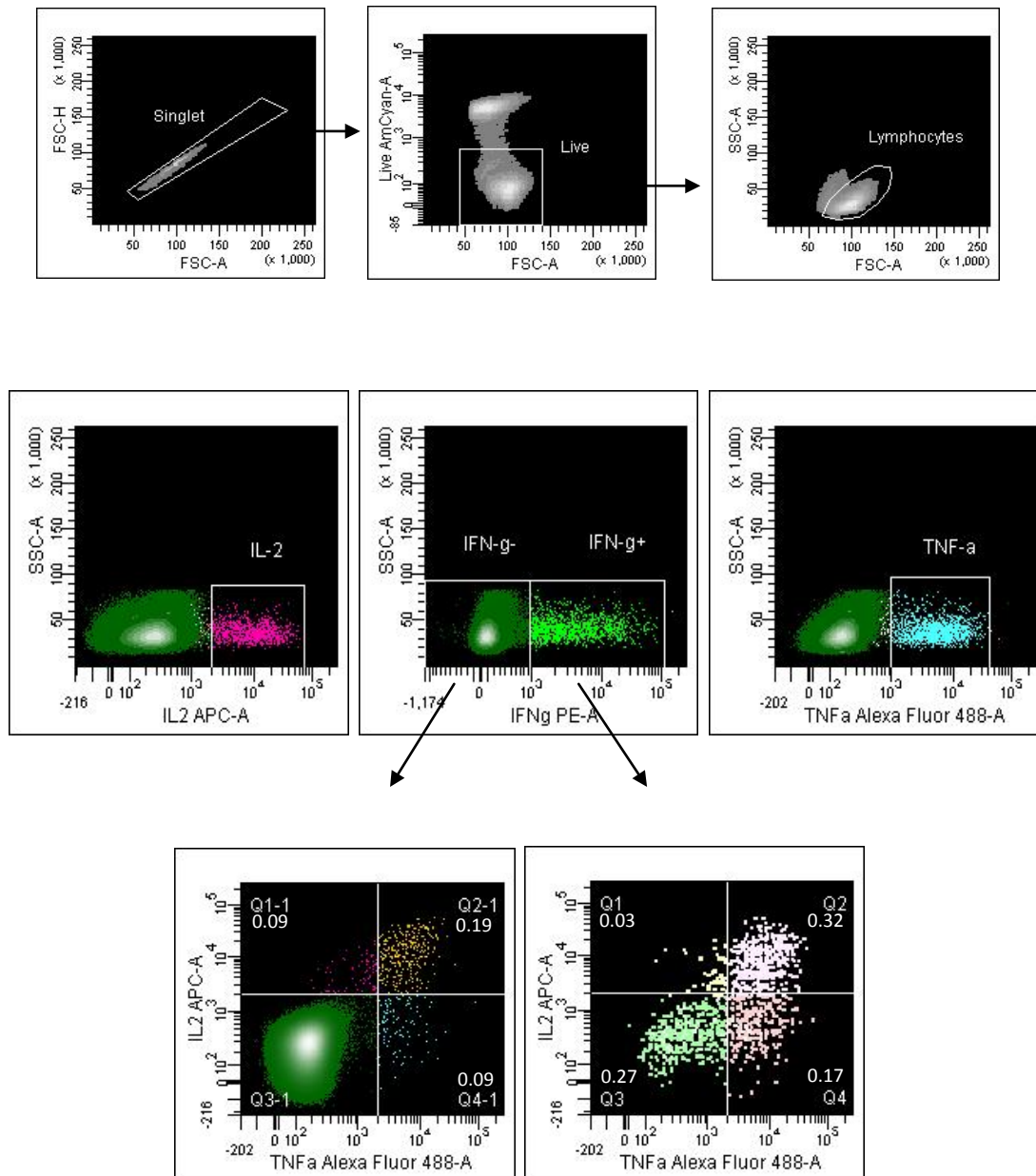


Figure 9: Identification of lymphocytes producing IFN- γ , IL-2 and TNF- α . PBMCs from experimental calves were isolated, stimulated with PPDj antigen, stained by intracellular cytokine staining (ICS) and interrogated by flow cytometry. Plots were gated on live lymphocytes and analyzed for all combinations of concurrent IFN- γ , IL-2 and TNF- α production. Numbers indicate percentage of lymphocytes in the seven individual cell subsets (Illustration of FACS plots for polyfunctional ICS, Aneesh Thakur).

In MAP-MSV-2 study, we also stimulated PBMCs at defined time points with vaccine antigen cocktail, superantigen and PBS for 4 hrs and froze the cells at -70°C in RLT buffer for future mRNA expression analysis.

Comparative intradermal tuberculin skin testing

Comparative intradermal tuberculin skin testing using bovine and avian PPD was performed on all the animals from both studies before slaughter. The skin thickness was recorded 72 hours after intradermal injection of PPDs, and results were interpreted according to guidelines of European Communities Commission regulation 141 number 1226/2002 (105). Additionally, protein and peptide cocktails containing ESAT-6, CFP10 and Rv3615c were used for skin testing to rule out cross-reaction with bovine TB. In MAP-MSV-2 study, we collected PBMCs from all animals prior to skin testing and froze down the PBMCs at -70°C in freezing mixture comprising of 10% dimethyl sulfoxide (DMSO) and 50% foetal calf serum (FCS) in RPMI medium.

Necropsy

In MAP MSV-2 experiment, the first eight born calves were euthanized and necropsied at 44 weeks and remainder 20 calves at 52 weeks of age. Fourteen tissue samples from each animal were collected separately in RLT buffer (for mRNA expression), sterile PBS (for *IS900* qPCR), and 10% neutral buffered formalin (for histopathology) and included: ileocaecal valve, ileum (0 cm, -25 cm, -50 cm, -75 cm; distance indicated relative to the location of ileocaecal valve in proximal direction), jejunum (-100 cm, -150 cm, -250 cm, -300 cm), and lymph nodes (ileocaecal lymph node; mesenteric lymph nodes corresponding to respective distances). Tissue samples in RLT buffer were frozen at -20°C for future analyses. For *IS900* qPCR, tissue samples were rinsed with sterile PBS. Epithelium, submucosa, and lamina propria were scraped from the serosa with sterile object glass. The tissue scrapings were then frozen at -20°C in 10 ml sterile PBS. Later, scrapings were homogenized by blending in a homogenizer,

centrifuged, weighed and processed for DNA extraction. Samples collected in formalin were embedded in paraffin wax and sectioned. Hematoxylin and Eosin (HE) and Ziehl-Neelsen staining was performed on the sections using standard histological methods. The analyses of these sections were not performed prior to submission of this thesis. We also collected ileocaecal lymph node and spleen tissues in Hank's balanced salt solution (HBSS) at necropsy from each animal and froze down the lymphocytes at -70°C in freezing mixture comprising of 10% DMSO and 50% FCS in RPMI medium.

Quantitative Real Time PCR (qPCR)

qPCR targeting MAP-specific *IS900* sequence was employed for the detection and quantification of MAP in faecal samples and tissues collected at necropsy. We followed the protocol as has been published earlier for the quantification of MAP in ovine faecal samples (69). Primers used in this paper are MAP-specific and targets the *IS900* sequence. However, we were unable to get efficient amplification with this primer set and calculations of melting temperature (T_m) gave 75.2°C for the forward and 65.9°C for the reverse primer. Because of the large difference in T_m we designed two new primers targeting the *IS900* sequence in MAP with T_m , 71.7°C and 72°C respectively for the forward (5'-GGCAAGACCGACGCCAAAGA-3') and reverse (5'-GGGTCCGATCAGCCACCAGA-3') primers and found high efficiency. Primers were designed using Primer 3 software (<http://frodo.wi.mit.edu/cgi-bin/primer3/primer3www.cgi>) (133).

For DNA extraction, tissue samples were homogenized and subjected to bead beating (Zirconia/Silica beads) followed by genomic DNA extraction using DNAeasy Blood and Tissue kit (Qiagen). DNA extraction from faecal samples was done using QIAmp DNA stool kit after bead beating. We didn't culture the faecal samples and tissues in our study due to long culturing time and poor sensitivity of MAP culturing. PCR is a highly sensitive technique compared to MAP culturing and has been demonstrated to quantify the absolute MAP numbers in clinical samples (68). In our study, we did a

relative quantification of MAP among faecal samples and tissues and compared the samples between the vaccinated and non-vaccinated groups to observe the protective effect of vaccine. For this we designed two standard curves, one for the tissue samples and another for faecal samples by spiking their extracted DNA with DNA from the reference MAP bacteria (MAP Ejlskov) in ten-fold dilutions. Tissue (jejunum at -250 cm) and faecal sample DNA from an animal found to be consistently negative through qPCR was used for spiking. By spiking the tissue and faecal DNA samples with known concentration (CFU) of bacterial DNA, we were able to make two standard curves and relatively quantify the bacterial number in all the samples. The analyses of faecal samples were not performed prior to submission of this thesis.

RESULTS

Article 1

Cell-mediated and humoral immune responses after immunization of calves with recombinant multi-antigenic MAP subunit vaccine at different ages

Neonates and juvenile ruminants are more susceptible to paratuberculosis infection. Thus if calf susceptibility to MAP infection is related to maturation of the immune system, the immune response to a MAP vaccine may also be influenced by the age of vaccination. The aim of the study was to evaluate the appropriate age of vaccination of the animals against a subunit vaccine comprised of five MAP recombinant proteins formulated with CAF01 adjuvant in order to generate a high frequency of antigen-specific T cells with more rapid effector functions. A significant IFN- γ production against the vaccine recombinant proteins in all three groups of vaccinated calves as compared to the adjuvant controls was observed. Among the five proteins, the most significant responses were observed against MAP-3, LATP-4 and ESAP-2. Vaccinated calves had IFN- γ levels below 50 pg/ml against both PPDj and PPDb antigens confirming the specificity of the vaccine-induced immune response. Among the three vaccine age groups, Vac16w had the most consistent IFN- γ responses post vaccination, although there were no significant differences in IFN- γ levels against vaccine proteins between the groups. Only exception was protein MAP-3, with significantly lower IFN- γ levels in group Vac8w. We couldn't associate IFN- γ producing capacity of the blood cells with the age of the animals when stimulated with superantigens. In addition, we didn't observe any significant increase in the IFN- γ levels after second booster vaccination in all three vaccine groups. The humoral IgG1 immune responses against vaccine recombinant proteins mostly mimicked IFN- γ responses. Most significant responses were observed against LATP-4, MAP-3, ESAP-2 proteins followed by Ag85B and SECP-1. Likewise, the second booster vaccination couldn't elevate the IgG1 responses against any of the five MAP proteins in three vaccine groups. In addition to the CMI and humoral immune responses, the effect of subunit vaccine and cross-reaction with bovine

tuberculosis was also assessed. No visible swelling or palpable growth or other side effects were noticed post vaccination. Comparative intradermal tuberculin testing confirmed all calves used in the study to be negative for bovine tuberculosis, which was supplemented by low whole-blood IFN- γ levels against PPD_b and PPD_a. Interpretation as a single tuberculin test also showed negative values in all but one calf.

Article 2

Increasing the *ex vivo* antigen-specific IFN- γ production in subpopulations of T cells by anti-CD28, anti-CD49d and recombinant IL-12 costimulation in cattle vaccinated with recombinant proteins from *Mycobacterium avium* subspecies *paratuberculosis*

In MAP infection, the inflammatory environment in the small intestine provides necessary costimulatory signals that might be missing on evaluating the IFN- γ responses in *ex vivo* PBMC assays. The aim of the study was to identify the effect of costimulation using signal 2 costimulatory molecule, anti-CD28 (aCD28) or anti-CD49d (aCD49d) and anti-CD5 (aCD5) mAbs, alone or in combination with a costimulatory signal 3 cytokine, recombinant IL-12 (rIL-12) on the frequency of T cells responding to MAP antigens in *ex vivo* PBMCs cultures in experimentally challenged and vaccinated calves. Both aCD28/aCD49d and aCD5/aCD28/aCD49d were found to significantly ($p < 0.01$) elevate the MAP vaccine antigen-specific IFN- γ production in PBMCs cultures compared to control animals. A significant interaction ($p < 0.05$) was observed between aCD28 and aCD49d mAb. Same cultures showed a further increase in the IFN- γ levels after addition of rIL-12 alone or rIL-12 with aCD28/aCD49d, though accompanied by background IFN- γ release in one of the control animals. Flow cytometry based integrated mean fluorescence intensity (iMFI) was measured to detect the enhanced IFN- γ production by signal 2 and signal 3 costimulatory molecules among CD4⁺, CD8⁺, $\gamma\delta$ T cells and NK cells. aCD28 was found to enhance more IFN- γ iMFI as compared to aCD49d or aCD5 mAb alone in all cell subsets. However, the significant increase in the IFN- γ iMFI with low non-specific background signal was observed with aCD28/aCD49d combined with strong effect of interaction ($p < 0.05$). rIL-12 with or

without aCD28/aCD49d potentiates IFN- γ production among all four subsets with strong increase in CD4+, CD8+ and $\gamma\delta$ T cells. This increase was, however, accompanied with an increased background in the control animals that was absent in case of aCD28/aCD49d costimulation. While the profile of IFN- γ producing cells after aCD28/sCD49d costimulation still reflected the profile without costimulation, the marked increase in the IFN- γ production of CD3+CD4+ T cells after addition of rIL-12, with or without aCD28/aCD49d, skewed the profile of IFN- γ producing cells towards a CD3+CD4+ T cell dominance.

Article 3

Immune markers and correlates of protection for vaccine-induced immune responses

Vaccine development is a cumbersome and lengthy process. The aim of the study was to review immune markers and new candidates for correlates of protective vaccine-induced immune responses against chronic infections and their possible role in defining the protective immunity in human and veterinary medicine. Well-defined and newly recognized immune markers and correlates of CMI and humoral immunity were extracted from literature and summarized in two tables. A third table summates the T cell immunoassays used to identify CMI based immune markers. While the different stages involved in the induction and regulation of CMI and humoral immune responses against pathogens has been represented through a figure. Almost all of the commercially available vaccines induce a robust antibody response that correlates with the level of protection. The neutralizing antibody titers for majority of the available vaccines along with their protective threshold values have been reported. Other recognized and potential immune markers and correlates of humoral immunity have been discussed as functions of ADCC, immunoglobulin class and subclass (IgG1, IgG2, IgA) and memory B cells citing references from many chronic infections. For intracellular infections with antibodies as poor correlate of protection, several characteristics of T cell such as phenotype, function, antigen specificity and MHC-restriction have been reviewed and discussed as potential correlates of protection. The role of T_H1, T_H2 and T_H17 cells, CTL and associated cytokines, particularly IFN- γ has been described as potential

immune correlates against chronic infections. MHC tetramers and multimers have been described in the light of their potential as immune markers and correlates for viral infections requiring CD8+ T cell response. Furthermore, central and effector memory T cells, polyfunctional T cells and innate effector cells such as iNKT and $\gamma\delta$ T cells have been detailed as proven and potential new candidates. Specific examples of existent and potential immune correlates and methods to analyze them for chronic infections in veterinary medicine have been explained. A perspective describing the potential of and challenge associated with identification of immune correlates especially in infections requiring CMI has been set forth.

Article 4

A novel multi-stage subunit vaccine against paratuberculosis induces significant immunity and reduces bacterial burden in tissues

Paratuberculosis is a staged infection. Effective vaccines are lacking for paratuberculosis control in cattle. We hypothesize that a vaccine incorporating proteins from different stages of infection could decrease MAP shedding and prevent reactivation of disease. The aim of the study was to develop a novel multi-stage paratuberculosis vaccine, FET11 and characterize the protective efficacy in MAP infected calves. A fusion of four early expressed MAP proteins and a latency-associated MAP protein were formulated with CAF01 adjuvant and injected to MAP challenged calves at two different age groups. Efficacy of FET11 vaccine was measured as a function of CMI responses to component vaccine proteins and relative bacterial burden in gut tissues (ileum and jejunum) as quantified by IS900 qPCR assay. Relative quantification of MAP load in the tissues from six different sites of gut revealed a mean 1.1 log₁₀ reduction in animals from late FET11 vaccine group compared to control. This decrease was correlated with IFN- γ levels in response to vaccine proteins at week 32. IFN- γ response to PPDj was correlated with higher bacterial load in non-protected animals at week 48 and 52. A differential immune response profile was observed among vaccine groups. Significant IFN- γ production against vaccine

proteins ESAP-2, ESAP-5 and LAMP-5 was observed in late FET11 vaccinated animals. Antigen-specific IFN- γ responses to latency protein, LAMP-5 were characteristically absent from control and Silirum[®] vaccinated animals. Two MAP proteins ESAP-2 and MAP-3, that were also present in the MAP MSV-1 experiment, show reduced immunogenicity in this study. None of the animals from the FET11 vaccine groups responded positively to comparative intradermal tuberculin test. While one animal each from control and Silirum[®] groups reacted to PPD_B and one of the Silirum[®] vaccinated animals was positive with skin test. Older animals developed a more robust antigen-specific immune response after FET11 vaccination.

DISCUSSION

FET11 vaccine

In article 4, we hypothesized that for a vaccine to be effective against paratuberculosis, it should incorporate proteins from different stages of infection and such a vaccine could decrease MAP shedding and prevent reactivation of disease. A similar approach in human tuberculosis has shown superiority of multi-stage vaccine, H56 over BCG vaccine in mice models (1). FET11 vaccine, developed from a fusion of four early expressed and a latency-associated protein formulated with CAF01 adjuvant induced significant antigen-specific IFN- γ responses in animals vaccinated at 16 weeks of age (Late FET11 vaccination). Late FET11 vaccination confers protection characterized by a mean 1.1 log₁₀ reduction in bacterial load in gut tissues in comparison with control group. This reduced bacterial burden however, did not correlate with corresponding whole-blood IFN- γ responses to all component vaccine proteins at all time points but at week 32 of the study. This discrepancy implies possible role of antibodies (74) or polyfunctional T cells in accordance with our hypothesis that needs to be validated. A differential immune response to early and late stage MAP proteins and consistent CMI responses in older animals complements our earlier findings from MAP MSV-1 study (Article 1). A correlation between IFN- γ responses to PPDj in advanced weeks of the experiment and relative bacterial burden in the tissues emphasize PPDj responses as a measure of infection status in experimental MAP infections. The delivery of selected candidates as a single fusion protein has the potential benefit of enhanced responses to low responder proteins such as MAP-3 and ESAP-3 in our study. Similar observation was found for low immunogenic ESAT-6 when used with antigen 85B in a strong adjuvant DDA-MPL (12, 178).

Age of vaccination and immune response to MAP

In article 1, we hypothesized that the age of calves when being vaccinated against MAP affects the vaccine- induced immune response. Results from this study showed reduced cell-mediated immune

responses when the animals were vaccinated at 8 weeks of age compared to those vaccinated at 2 or 16 weeks of age. The 8 weeks time point correlates with dietary stress from the calves being weaned off milk. The humoral response seems independent of this as we saw no difference in the three vaccination groups in terms of MAP vaccine-induced antibody responses. In contrast, findings from a recent study suggested that ingestion of colostral antibodies interfere with the immune capacity of the young calves to produce antigen-specific antibody responses, with little or no effect on cell-mediated immune response (115). Among the three vaccine age groups, animals vaccinated at 16 weeks of age had the most consistent IFN- γ responses against vaccine proteins (Article 1 and 2). Based on the findings presented in article 1, we did not consider vaccination of calves at 8 weeks of age in MAP MSV-2 experiment (Article 4). It seems that dietary stress could jeopardize the immune response and vaccination of older calves appears to result in more sustained CMI responses (Article 1). Thus the animal age at vaccination should be considered when designing vaccination experiments in calves. No experimental MAP challenge was performed in this study (MAP MSV-1). Nevertheless, considering the pathogenesis of MAP infection in cattle, analyzing the establishment of infection in young calves combined with age of vaccination could shed light on the methods to improve development of a strong immunity against the disease. We used this information to design early and late FET11 vaccination groups in MAP MSV-2 experiment.

Acute and latent stage MAP infection proteins

Our results demonstrate that MAP proteins expressed in different stages of infection have different immune response profiles following vaccination (Article 1 and 4). We surmised that slow pathogenesis of paratuberculosis can be described as an initial acute stage followed by a latent stage and that protein expression profile changes between the stages with some genes being relatively higher expressed in one or the other stage. This multi-stage vaccine strategy (H56 vaccine) has been shown to efficiently contain late stage human tuberculosis infection in mice models as compared to Ag85B-ESAT6 (H1) and BCG

vaccine (1). We observed significant higher IFN- γ as well as IgG1 levels against *Esx*-secretion system proteins, ESAP-2 and MAP-3 (Article 1), ESAP-2 and ESAP-5 (Article 4) and latency-associated proteins LATP-4 (Article 1) and LATP-5 (Article 4). However, proteins SECP-1 and Ag85B that are supposedly highly immunogenic owing to their extracellular environment were found to be poorly immunogenic (Article 1). Despite the established role of Ag85B in early proliferative and IFN- γ responses in natural and experimental tuberculosis infection in cattle and mice (131), its role in the protection against MAP infection when used as a recombinant subunit vaccine still remains to be elucidated. Significant and consistent immune response to *Esx*-secretion system and latency proteins in this study (Article 1 and 4) supports their role as potential candidates for a MAP subunit vaccine. The results also showed different immunogenicity for two MAP proteins, ESAP-2 and MAP-3 common in both MAP MSV-1 and MAP MSV-2 studies, which emphasizes the possibility of dynamics of MAP infection in guiding the differential immune response.

T cell signaling and costimulatory molecules

We have demonstrated in article 2 that costimulation through aCD28/aCD49d antibodies potentiate an antigen-specific production of IFN- γ against recombinant MAP proteins in *ex vivo* recall responses in different T cell subsets. This increase was accompanied by an increase in the iMFI of activated T cells. The study also shows the degree of signaling in T cells is regulated by the costimulatory environment. The inflammatory environment at the site of infection provides the necessary costimulatory signals that are critical in shaping the extent and nature of the immune response (24, 149). These signals might be missing when evaluating the peripheral immune responses as in the whole-blood or PBMC leading to a deficit of immune response. This deficit can be compensated by adding costimulatory molecules (signal 2 and 3) to the *ex vivo* T cell assays and has been routinely used in mice and human immunology (43, 173). Development of bovine anti-CD28 antibody allowed us to verify the use of this molecule as signal 2 costimulation in bovine T cell immunoassays. In combination with anti-CD49d, anti-CD28 costimulatory

molecule increased antigen-specific IFN- γ production with very low background. However, using rIL-12 (a signal 3 costimulatory molecule) alone or with signal 2 molecules showed the maximum antigen-specific IFN- γ production, though accompanied by a high non-specific response (Article 2). This observation highlights the bias towards a Th1 type immune response with rIL-12 as compared to a balanced or less biased response with signal 2 molecules, anti-CD28/CD49d.

A large number of cell surface signaling molecules have been identified to play a critical role in the control of immune responses (200). Modulation of these signaling molecules is one of the strategies adopted by pathogens to establish their existence in the host (73). Observations from article 2 define an important role for signaling molecules in MAP vaccine-induced immune responses. These findings offer the possibility of using signal 3 costimulatory molecules for supporting antigen-specific responses in diagnostic assays and signal 2 molecules to study the antigen-specific responses during e.g. an ongoing infection or vaccination over time.

Role of CD8+ T cells, $\gamma\delta$ T cells and NK cells in MAP vaccine-induced immune responses

IFN- γ is a key cytokine in protective immune responses against mycobacterial infections including MAP. The role of CD8+ T cells, $\gamma\delta$ T cells and NK cells in MAP vaccine-induced immune response as producer of IFN- γ has been demonstrated in article 2. Though the CD4+ T cells were the major producers of IFN- γ , CD8+ T cells and innate immune cells also contribute to the antigen-specific IFN- γ levels. CD8+ T cells have been found to play a role in the control of *M. tuberculosis* infection through different mechanisms such as IFN- γ release, cytotoxicity via granule-dependent exocytosis pathway, cytotoxicity mediated through Fas/Fas ligand interaction, and direct microbicidal activity (88). For the CD8+ T cells to produce IFN- γ , antigens must be presented on the MHC class I molecules through cross-presentation with macrophages or DCs. Thus we can rightfully speculate here that sufficient cross-presentation did occur in our *in vitro* cultures.

It is known that neonatal calves have significantly higher levels of circulating innate immune cells, NK cells and $\gamma\delta$ T cells, than adult cattle (83). Each of these cell populations can interact with mycobacteria infected antigen presenting cells and secrete high level of IFN- γ (117, 127). We also found IFN- γ production by these innate immune cells against MAP recombinant proteins induced recall responses following vaccination (Article 2). Neonatal vaccination with BCG vaccine confers significant protection in cattle and is used widely in human infants (146). Relative immaturity of the neonatal immune system is compensated by increased numbers of these innate immune effector cells that enables effective immune response to vaccination by secretion of high levels of IFN- γ (146). In addition to production of IFN- γ , NK cells and $\gamma\delta$ T cells execute their effector functions against mycobacterial infections by production of cytokines such as IL-17, IL-22 (34) as well as killing through perforin and granulysin (35). Detailed role of both these innate immune cells in MAP infection still remains to be fully elucidated.

Vaccine immune correlates

Based on our hypothesis that polyfunctional T cells will be an immunological correlate of vaccine-induced protection and curiosity to identify other immunological correlates, we conducted a literature review to explore the current knowledge about immune markers and correlates of protection for vaccine-induced immune responses (Article 3). Vaccination and infection-induced immune markers and correlates of cell- and antibody-mediated immunity were studied, with special emphasis on chronic intracellular infections. Presently, all the well-defined immune correlates for the commercially available vaccines belong to antibody-mediated immunity (126). Majority of these antibody-mediated immune correlates have been outlined for antiviral vaccines due to strong humoral responses in viral infections. For CMI based immune correlates, various recognized and potential immune markers were discussed. Findings from the article 3 largely support our hypothesis. However, for chronic intracellular infections in which

cell-mediated immunity plays an important role, identifying the appropriate immune correlates still remains to be validated.

Immune correlates of antibody-mediated immunity have been described for many chronic infections as a function of neutralization titer, ADCC, immunological class and subclass and memory B cells. Serum neutralizing antibody titers measured through ELISA and neutralization assays has been well-defined for many viral infections (125). FcγR mediated effector functions including ADCC have been found to be a better correlate of protection for viral infections including HIV. Likewise, serum and mucosal IgG and IgA levels and memory B cells have been associated with vaccine-mediated protection in many viral infections (126). Regarding the immune correlates of cell-mediated immunity for chronic intracellular infections, IFN-γ production by CD4⁺ T cells has been the most studied and recognized immune correlate. However, new findings challenge the role of IFN-γ in protection (42). Instead, other effector cells and cytokines of CMI have been invariably linked with protection following vaccination in various infectious disease setups. Simultaneous production of cytokines IFN-γ, TNF-α and IL-2 at single cell levels by CD4⁺ and CD8⁺ T cells referred to as polyfunctional or multifunctional T cells has been increasingly correlated with protection following vaccination and infection (30, 90, 142). In addition, T_H17 cells, CTL, memory T cells (central and effector), MHC tetramers, cytokines and chemokines such as IL-1, IL-10, IL-12, IL-17, IP-10, TGF-β, granulocyte macrophage colony-stimulating factor (GM-CSF) have been elaborated as potential immune correlates in the light of available evidence from many chronic infections. Innate immune cells such as NK cells, NKT cells and γδ T cells and their effector cytokines have been linked to various extents with vaccine-mediated protection.

For large animal species, there is a major focus on measuring IFN-γ response as an immune correlate in vaccination studies. One of the challenges is the limited immunological toolbox in veterinary sciences. In cattle, the possibility of measuring polyfunctional T cells has only recently been demonstrated with the

development of bovine IL-2 antibody (183). Likewise, development of peptide-MHC tetramers in pigs has allowed monitoring CD8+ T cells responses post vaccination (122). In addition, gene expression studies have allowed evaluating many cytokines, chemokines and effector T cell responses (44). Thus with the expansion of available immunological reagents and techniques, specific immune markers and correlates of protection for diseases of veterinary importance should be expected in near future. Based on the knowledge gained from this review, we continued to explore the role of CMI based immune responses chiefly IFN- γ production by CD4+ T cells in our MAP challenge and vaccination studies keeping in mind the possible role of other immune cells especially $\gamma\delta$ T cells, CD8+ T cells and polyfunctional T cells. In addition, humoral immune responses were also measured as a possible correlate. We also measured the CFSE proliferation, expression of activation markers, CD25, CD44, CD62L and chemokine receptor CCR7 in our study to identify possible vaccine immune correlates.

CONCLUSIONS AND PERSPECTIVES

In this thesis, information about the development of a multi-stage subunit vaccine against paratuberculosis infection was obtained through experimental infection studies in calves. The results also accentuates consideration of age for vaccination against MAP, costimulation signals for measurement of T cells responses, role of CD8+ T cells, $\gamma\delta$ T cells and NK cells in MAP infection/vaccine immune responses and possible immunological markers and correlates of protection.

The results show that vaccination with multi-stage vaccine, FET11 at 16 weeks of age induces significant immune response and confers protective immunity characterized by a mean 1.1 log₁₀ reduction in bacterial load in gut tissues compared to control animals. These findings are important due to the fact that existing MAP vaccines do not significantly reduce bacterial burden in infected animals. The protective efficacy of the late FET11 vaccination correlated well with the IFN- γ responses to vaccine proteins about 12 weeks after vaccination. However, we saw a decrease in the IFN- γ responses to component vaccine proteins after one year. Therefore, a booster vaccination at this stage could be useful to amplify the immune response. It would be of interest to investigate the protective efficacy of FET11 vaccine in experimental animals over an extended period of time exceeding one year. In addition, an evaluation of the performance of FET11 vaccine over a number of years in a natural MAP infected cattle herd is needed, as this could be a much better method to access the level of protection or decreased faecal shedding in animals at different stages of the disease as well as to rule out reactivation of infection. In the one-year time frame, early FET11 vaccination performed poorly. Likewise, commercial Silirum® vaccine did not show significant protection. More studies are required to address their performance over a longer period of time. Our results also highlight the application of qPCR for the evaluation of microbial load in tissues and vaccine efficacy.

Experimental infection of young calves using mid-log-phase frozen stock MAP cultures and correlation of infection with PPDj responses in this study highlights the possibility of establishing a uniform and reproducible bovine MAP infection model despite non-availability of age matched calves in desired numbers at the same time for vaccine experiments. In addition, vaccine-induced immune responses were influenced by the age of vaccination of calves with poor responses when vaccination coincided with the age of weaning. Both MAP MSV-I and II studies, support that older animals (4 months) develop a more robust immune response, even when infection occurs at an earlier age. The results showed that proteins expressed at different levels during various stages of MAP infection induce different immunological profile when used with CAF01 adjuvant as a subunit vaccine, with significant immune responses against *Esx*-secretion system and latency proteins. Differential immune response against two MAP proteins common in both MSV experiments emphasizes the possibility of dynamics of MAP infection guiding the vaccine-induced immune response. Investigation of more candidate proteins with over expression in different stages of MAP infection and playing a key role at the host-pathogen interface, metabolism, and persistence could help decide optimal selection of components of a multi-stage subunit vaccine inducing stronger immunity.

CD8⁺ T cells and innate immune cells including $\gamma\delta$ T cells and NK cells appear to be important antigen-specific IFN- γ producers following vaccination in addition to already well-recognized CD4⁺ T cells. Future studies should examine the detailed role of $\gamma\delta$ T cells and NK cells in MAP infection and vaccine-induced immune responses.

Costimulation of T cell subsets by anti-CD28 and anti-CD49d antibodies was observed to enhance vaccine antigen-specific IFN- γ production. Recombinant IL-12 also increased IFN- γ levels but leads to a strong T_H1 bias and thus appears more useful for diagnostic purposes than to characterize the

contribution of all T cells to the vaccine-induced immune response. It would be interesting to investigate the costimulation effect on combined cytokine profiles such as polyfunctional T cells.

The significant IFN- γ responses to MAP vaccine proteins in our study may supplement the common notion of role of IFN- γ as an immunological marker of protection. However, we didn't find a strong correlation between IFN- γ responses to all component proteins and reduction in bacterial load in tissues at all time points throughout the study. One speculation could be that IFN- γ is not an ideal measure of protection and rather antibodies have an important role as a possible correlate, which need to be examined. We were able to identify polyfunctional T cells in our study. These results rouse further investigations to determine the significance of these cells as possible correlates of protection or infection or memory generation, for instance by looking at the cryopreserved lymphocytes from animals with differential bacterial burden within late FET11 group as well as comparing them with animals from other groups. This analysis may spotlight the possible duration of and type of memory cells induced by FET11 vaccine.

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Cell-mediated and humoral immune responses after immunization of calves with recombinant multi-antigenic MAP subunit vaccine at different ages

Aneesh Thakur,¹ Claus Aagaard,² Anders Stockmarr,³ Peter Andersen,² and Gregers Jungersen^{1*}

Adaptive Immunology and Parasitology, National Veterinary Institute, Technical University of Denmark, Bülowsvej 27, 1790 Copenhagen V, Denmark¹; Department of Infectious Disease Immunology, Statens Serum Institute, Artillerivej 5, 2300 Copenhagen, Denmark²; Data Analysis Section, Department of Informatics and Mathematical Modeling, Technical University of Denmark, Asmussens Alle, Building 305/126, 2800 Lyngby, Denmark³

* Corresponding author: Gregers Jungersen

National Veterinary Institute, Technical University of Denmark

Bülowsvej 27, 1870 Frederiksberg C, Denmark

Phone: +45 35 88 62 34

Fax: +45 35 88 66 01

E-mail: GRJU@vet.dtu.dk

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Aneesh Thakur,¹ Claus Aagaard,² Anders Stockmarr,³ Peter Andersen,² and Gregers Jungersen^{1*}

Adaptive Immunology and Parasitology, National Veterinary Institute, Technical University of Denmark, Bülowsvej 27, 1790 Copenhagen V, Denmark¹; Department of Infectious Disease Immunology, Statens Serum Institute, Ørestads Boulevard 5, 2300 Copenhagen, Denmark²; Data Analysis Section, Department of Informatics and Mathematical Modeling, Technical University of Denmark, Asmussens Alle, Building 305/126, 2800 Lyngby, Denmark³

* Corresponding author. Mailing address: Adaptive Immunology and Parasitology, National Veterinary Institute, Technical University of Denmark, Bülowsvej 27, 1790 Copenhagen V, Denmark.
Phone: +45 35 88 62 34. Fax: +45 35 88 66 01. E-mail: GRJU@vet.dtu.dk.

ABSTRACT

Neonates and juvenile ruminants are very susceptible to paratuberculosis infection. This is likely due to a high degree of exposure from their dams and an immature immune system. To test the influence of age on vaccine induced responses a cocktail of recombinant MAP proteins (SECP-1, ESAP-2, LATP-4, MAP-3 and Ag85B) was formulated in a cationic liposome adjuvant (CAF01) and used to vaccinate animals of different age. Male jersey calves were divided into 3 groups that were vaccinated at 2, 8 or 16 weeks of age and boosted twice at week 4 and 12 relative to the first vaccination. Vaccine induced immune responses, the IFN- γ cytokine secretion and antibody responses were followed for 20 weeks. In general, the specific responses were significantly elevated in all three vaccination groups after the first booster vaccination with no or only minor effect of the second booster. However, significant differences were observed in the immunogenicity of the different proteins and it appears that the older age group produced a more consistent IFN- γ response. In contrast, the humoral immune response is seemingly independent of the vaccination age as we found no difference in the IgG1 responses when comparing the three vaccination groups. Combined, our results suggest that appropriate age of vaccination should be considered in vaccination protocols and there is a possible interference of vaccine induced immune responses with weaning (week 8).

INTRODUCTION

Paratuberculosis is a chronic non-treatable granulomatous enteritis affecting all domestic and non-domestic ruminants and some non-ruminants worldwide. It is an economically significant and widespread disease of the ruminants caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP). Neonates and juvenile animals are most susceptible to infection which progresses through subclinical, clinical and advanced clinical disease stages (14, 39).

Currently available vaccines against MAP consist of killed or attenuated MAP in an oil adjuvant, which has been reported to provide partial protection through delayed fecal shedding and reduction in clinically affected animals (27, 37). Whole-cell based vaccines, however, induce production of antibodies and a sensitization to delayed type hypersensitivity (DTH), making it impossible to differentially diagnose naturally infected from vaccinated animals. Vaccination against MAP also interferes with the diagnosis and surveillance of bovine tuberculosis due to false-positive skin test results (38). Moreover, whole-cell-based live vaccines suffer with lack of characterization, localized prolonged swelling and granuloma formation at the site of injection and potential side effects on accidental self-inoculation among veterinarians while vaccinating animals (12, 13, 26).

Subunit vaccination with identified protective protein antigens in combination with an adjuvant inducing strong Th1-type immune responses could be an ideal strategy to surmount the limitations associated with whole-cell-based vaccines (10, 10, 29). Recently, paratuberculosis experimental vaccines based on recombinant proteins expressing mycobacterial antigens (11, 21, 28, 30, 37), DNA vaccines (30) and expression library immunizations (9) have been found to induce partial protection against experimental infection with MAP. Immaturity of the neonatal immune system contributes to an increased susceptibility of the young animal to infectious disease and may limit its capacity to develop a protective response to vaccination (22). Cattle usually become infected with MAP as calves, either

through in-utero transmission or as neonates via ingestion of fecal material, milk or colostrum containing MAP organisms (5, 34, 36). Newborns have a tendency to exhibit a Th2 profile (19) and paratuberculosis infection is characterized by a distinct Th1 response (33). If calf susceptibility to MAP infection is related to maturation of the immune system, the immune response to a MAP vaccine may also be influenced by the age of vaccination. Evaluating the appropriate age of vaccination of the animals against MAP is therefore important in order to generate a high frequency of antigen-specific T cells with more rapid effector functions. We hypothesized that age of vaccination influences the quality of MAP vaccine induced T-cell responses. In order to substantiate this hypothesis, we vaccinated calves with well-defined MAP recombinant proteins at 2, 8 or 16 weeks of age followed by two booster rounds 4 and 12 weeks after the first vaccination. Vaccine induced IFN- γ release and antibody responses were analyzed in individual animals to assess the immunogenicity of the vaccine. The upregulation of these immune responses were prospectively correlated with the age of the calves.

MATERIALS AND METHODS

Animals. A total of 27 male jersey calves were used in the present study. The animals were obtained at the age of 2 weeks from a farm which by the Danish paratuberculosis surveillance program had a true prevalence equal or close to zero at all milk-antibody samplings from September 2006 to January 2011 (17). Animals were housed and raised under appropriate biological containment facilities (BSL-2) in a community pen with straw bedding. Calves were fed commercial milk replacer (DLG, Denmark) and green start (DLG, Denmark) for the first two months twice a day. At 8 weeks of age, calves were weaned and fed pellets and *ad lib* hay for the remainder of the study period. Calves had access to *ad lib* water and bedding was changed every day. Calves were checked daily for general health.

The use of animals described in this experiment was approved by the Danish National Experiments Inspectorate and carried out in compliance with their regulations and policies.

Antigens. Antigens in the vaccine are detailed in table 1. They were selected based on their immunogenicity as studied in naturally infected heifers and representing one protein from each of the Secreted proteins (SECP-1), Latency proteins (LATP-4), and ESAT-6 family member proteins (ESAP-2) along with a hypothetical protein (MAP-3) as reported earlier (17). Ag85B was included as a positive control

TABLE 1. Details of antigens used in the study

Antigen	Homology in MAA (%)	Homology in <i>M. bovis</i> (%)	Protein size (amino acids)	Product of MAP
SECP-1	99	83	300	Secreted protein
Ag85B	99	85	330	Secreted antigen 85B (Ag85B)
ESAP-2	100	87	98	Hypothetical ESAT-6 like protein
LATP-4	100	71	146	Heat shock protein (latency protein)
MAP-3	100	84	97	Hypothetical antigen

antigen based on earlier studies reporting significant IFN- γ responses in cattle and mice to Ag85B component of MAP (21, 28). All antigens were produced in *E. coli* and purified by metal affinity and anion columns as previously described (17). The protein concentration of the final products was measured by NanoOrange® protein quantification kit (Invitrogen).

Experimental design. Calves were distributed into three groups Vac 2w, Vac 8w and Vac 16w with 9 calves in each group. Each group comprised of 7 vaccine calves and two randomly assigned adjuvant controls. Calves were born over a period of 4 months and assigned into groups as they were born i.e. group Vac 16w includes the 9 oldest animals followed by Vac8w and Vac 2w. All vaccine calves in the three groups received 100 μ g of each recombinant vaccine antigen SECP-1, ESAP-2, LATP-4, MAP-3 and Ag85B formulated with the DDA/TDB (CAF01) adjuvant (2500 μ g/500 μ g) (3). After mixing, the vaccine antigens were allowed to adsorb to the adjuvant CAF01 for 1 hour at RT before injection. Controls received the adjuvant only along with sterile PBS. Vaccinations were performed through sub-cutaneous

route in the right mid-neck region 7 cm ahead of the prescapular lymph node. The age at the first vaccination in the groups Vac 2w, Vac 8w and Vac 16w was 2, 8 and 16 weeks, respectively. All vaccine calves were vaccinated three times with revaccinations at week 4 and 12 relative to the first vaccination (Fig. 1). Blood samples were collected every 2 weeks throughout the experiment. Heparinized samples were used for whole blood IFN- γ assay and serum samples were taken for serological analysis.

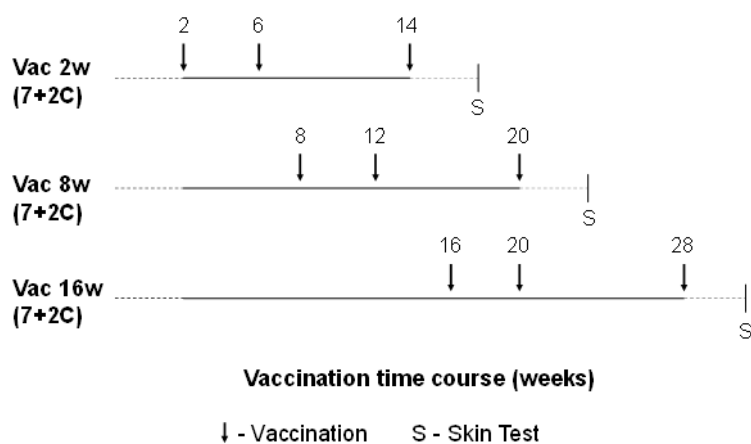


FIG. 1. Experimental design. Within each age-group of 7 MAP vaccinated animals, 2 calves received the CAF01 adjuvant only.

IFN- γ assay. Heparinized whole-blood samples were collected by the vacutainer system. 0.5 ml whole blood was stimulated in 48-well culture plates (Greiner Bio-One, Heidelberg, Germany) with previously added purified protein derivative Johnin (PPDj), purified protein derivative bovine (PPDb) and avian (PPDa) (a kind gift from James McNair, AFBI, Belfast), each of the five MAP vaccine antigens, or PBS and two positive control stimulations with phytohaemagglutinin (PHA; Sigma) or the superantigen *Staphylococcus* enterotoxin B (SEB; Sigma), respectively (50 μ l volume). All MAP antigens, PPDb, PPDa and SEB were added to a final concentration of 1 μ g/ml, while PHA and PPDj were added to a final concentration of 5 μ g/ml and 10 μ g/ml, respectively. Whole blood cultures were incubated for 18-20 h at 37°C and 5% CO₂ in air. Following overnight incubation, 55 μ l heparin solution (10 IU/ml in blood) was added to avoid clots in the supernatant after freezing. Plates were then centrifuged and approximately

0.35 ml of supernatant was collected into 96-well 1-ml polypropylene storage plates (Greiner Bio-One, Heidelberg, Germany) and frozen at -20°C until analysis.

The antigen-specific IFN- γ secretion in supernatants was determined by use of an in-house monoclonal sandwich ELISA as described earlier (18). The levels of IFN- γ (pg/ml) were calculated using linear regression on log-log transformed readings from the two-fold dilution series of a reference plasma standard with predetermined IFN- γ concentration. The IFN- γ response to PBS was subtracted from IFN- γ response to each of five recombinant MAP antigens among all the calves and defined as the antigen-specific IFN- γ response.

Antigen-specific serum IgG1 ELISA. An in-house developed and optimized indirect enzyme-linked immunosorbent assay (ELISA) was applied for the demonstration of serum IgG1 antibody responses against the five vaccine recombinant proteins throughout the study. Serum IgG2 levels were also measured but were found to be very weak for any useful interpretation and thus have not been discussed further. Briefly, microtiter plates (MaxisorpTM, Nunc A/S, Roskilde, Denmark) were coated overnight with 100 μ l of 1 μ g/ml of each of the five MAP antigens in Sodium carbonate buffer except for LATP-4 for which 0.2 μ g of coating concentration was used. Next day plates were blocked using 200 μ l PBS with 0.05% Tween-20 and 1% bovine serum albumin (PBS-T-BSA). The plates were then washed with PBS with 0.05% Tween-20 (PBS-T) and sera in a final dilution of 1:40 in PBS-T-BSA were added to test wells in duplicate and incubated at RT in a shaking incubator for 1 h. To allow for plate-to-plate calibration, Positive (PosC) and negative (NegC) control sera, added to test wells in triplicate, along with blank (PBS-T-BSA) were included on each plate. PosC sera were the sera from a vaccinated calf with consistent higher antibody responses against all vaccine antigens collected at 4 weeks after second vaccination (V2), and 2 and 4 weeks after third vaccination (V3) and following an exponential curve. NegC sera were the sera from the same calf on the day of vaccination (Day 0). The plates were washed

166 with PBS-T buffer and incubated with 100 µl 1:500 dilution of horseradish peroxidase-conjugated mouse
 167 anti-bovine IgG1 (Clone IL-A60; AbD Serotec) for 1 h at room temperature. After washing with PBS-T
 168 buffer, substrate (OPD, 1, 2-orthophenyl-diamine; H₂O₂) was added to each test well, and the
 169 absorbance was read at 493 nm with 649 nm reference subtraction. The mean OD value for each test
 170 serum (OD_{Sample}) was calibrated according to the OD-values of the control sera on the respective plate
 171 and presented as calibrated OD (OD_{Cal})

$$172 \quad OD_{Cal} = \frac{(OD_{Sample} - OD_{NegC}) \times (OD_{Pnorm} - OD_{Nnorm}) + OD_{Nnorm}}{(OD_{PosC} - OD_{NegC})}$$

174 where

175 OD_{Sample} is the sample OD

176 OD_{PosC} is the mean of positive controls on the plate (mean OD of three sera all in triplicate)

177 OD_{NegC} is the mean of negative control on the plate (mean OD of Day 0 sera in triplicate)

178 OD_{Pnorm} is the mean over all runs of positive controls

179 OD_{Nnorm} is the mean over all runs of negative controls

180

181 **Tuberculin skin test.** To evaluate the development of the cell-mediated immune response and to
 182 negate the possibility of any cross-reactivity with surveillance testing for bovine tuberculosis, tuberculin
 183 skin testing was performed 6 weeks after the third immunization. The single comparative intradermal
 184 tuberculin test with avian and bovine purified protein derivative (PPD-A and PPD-B respectively; 2000 IU
 185 each) was performed by intra-dermal inoculation of 0.1 ml of PPD-A and PPD-B (a kind gift from James
 186 McNair, AFBI, Belfast) and reactions were read 72 hours later. Results were recorded as increase in skin
 187 thickness at 72 hours compared to thickness before injection and interpreted according to the standard
 188 protocol (European Communities Commission regulation 141 number 1226/2002) (20). Reactions to
 189 each of the tuberculin's were interpreted as follows: a skin test reaction was considered positive when

skin thickness increased 4 mm or more, inconclusive when there was an increase of more than 2 mm and less than 4 mm, and negative when the increase was not more than 2 mm.

Following the guideline to the official interpretation of the intradermal comparative cervical tuberculin test an animal was scored positive if the increase in skin thickness at the bovine site of injection was more than 4 mm greater than at avian site, inconclusive if the increase in skin thickness at the bovine site was 1 to 4 mm greater than the avian reaction, and negative if the increase in skin thickness at the bovine site of injection was less than or equal to the increase in the skin reaction at the avian site of injection.

Statistical analysis and interpretation. For the statistical analyses of the antigen-specific immune responses the adjuvant control animals were pooled into one group. In order to obtain a single value of the immune response following each vaccination, the mean of the IFN- γ values obtained 2 and 4 weeks after each vaccination was used. For each antigen, data were analyzed with a zero-inflated model due to the excess amount of data registered with a 0 measurement. The probability that an IFN- γ measurement was positive was modeled (model A) with a logistic regression model where vaccine group (Vac 2w, Vac 8w, Vac 16w or Control) and Vaccination number (1, 2 or 3) were entered as deterministic explanatory variables, while an animal effect was included as a random effect. In order to obtain normality distributions, the responses above 2 pg/ml were log-transformed and modeled (Model B) with a mixed linear model with the same set of explanatory variables, and the two models were combined into one zero-inflated model (Model C). A statistically significant immunological response to the individual antigen was defined as a statistically significant effect in Model C ($p < 0.05$). Tests were performed with the likelihood method, using the Wald test (4). For MAP-3, the number of positive values were so different between vaccinated and non-vaccinated animals, that a logistic regression model (A) and thus a zero-inflated model (C) did not apply. Instead, a non-parametric Wilcoxon test (15) was used. This was

modified to the Kruskal-Wallis rank test (15) when comparing more than two groups. For each antigen, the corresponding antibody responses were modeled with a model similar to model B. Log-transformation of OD_{cal} values were performed for all antibody responses except for antigen SECP-1 which were normally distributed. Identical analyses were also performed using only results after the first two vaccinations to assess the immune response following a two-dose regimen. All analyses were carried out in Splus[®], version 6.1.

RESULTS

Observations following vaccination. The animals were observed throughout the study for any visible effects post vaccination. However, no side effect, such as swelling or palpable growth was observed following mid-neck subcutaneous inoculation of the recombinant proteins formulated in CAF01 adjuvant or by adjuvant alone.

Cell-mediated immune responses to vaccine antigens. IFN- γ release towards each of the five antigens in the vaccine was followed in individual animals for 20 weeks (Fig. 2). In general, adjuvant control calves did not show antigen-specific response to the vaccine antigens. However, the heat-shock protein LAMP-4 induced some IFN- γ responses in all adjuvant controls and one calf responded to several of the other antigens at the first sampling prior to vaccination and at two samplings after the third vaccination.

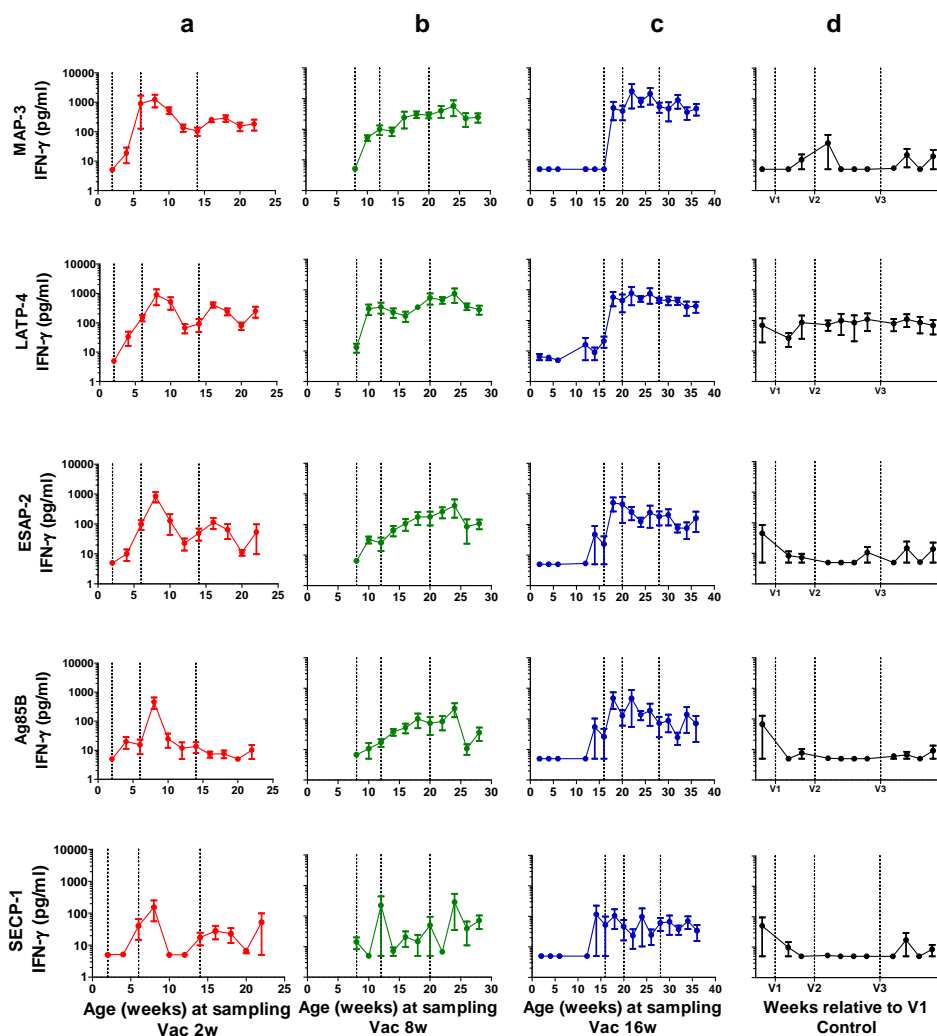


FIG. 2. Antigen-specific IFN- γ responses in whole-blood cultures. (a to d) Levels of IFN- γ released from whole-blood cultures stimulated with MAP recombinant antigens from calves in groups Vac 2w (a), Vac 8w (b), Vac 16w (c) and adjuvant control (d) following vaccinations indicated as dotted lines. (i to v) Whole-blood IFN- γ responses to stimulation with MAP recombinant proteins. IFN- γ levels are expressed as mean values (\pm standard deviation [SD]) for plasma concentrations (pg/ml). Statistically significant immune responses to all the vaccine antigens were observed following vaccination compared to adjuvant controls (table 2). The most significant responses were observed against MAP-3, LATP-4 and ESAP-2, while responses against Ag85B and in particular SECP-1 were less

significant. Comparison of the response levels to different vaccine antigens confirmed that MAP-3 and LATP-4 induced significantly higher levels than other antigens. Vaccinated calves had IFN- γ levels below 50 pg/ml towards PPDj and PPDb antigens (data not shown).

TABLE 2. P-values and parameter estimates – Full zero-inflated model (IFN- γ)

Antigen	Vac 2w vs. control	Vac 8w vs. control	Vac 16w vs. control	Vaccination response at three vaccinations equal?	Vaccination at all time points vs. control	Effect of Age
SECP-1	p=0.13	p=0.18	p=0.006**	YES p=0.28	p=0.04*	p=0.64
Ag85B	p=0.30	p=0.003**	p=0.005**	YES p=0.15	p=0.005**	p=0.68
ESAP-2	p=0.0009***	p=0.02*	p<0.0001***	YES p=0.55	p<0.0001***	p=0.08
LATP-4	p<0.0001*** [◊]	p<0.0001*** [◊]	p<0.0001*** [◊]	YES p=0.70 [◊]	p<0.0001***	NA
MAP-3	p<0.0001*** [◊]	p<0.0001*** [◊]	p<0.0001*** [◊]	NO p=0.02 [◊]	NA	p=0.13 [◊]

◊: All non-controls were positive. For group Vac 2w and Vac 16w (MAP-3), the difference is significant w.r.t. level of response (p=0.03 and 0.01). For LATP-4, all controls except 3 were positive; one at vaccination number 2 and two at vaccination number 3. For LATP-4, the p-value on time for positive level is thus based on a very small sample; which makes it unreliable.

From fig. 2 it appears that immune responses in group Vac16w were more consistent than responses in younger age-groups. However, no statistically significant differences in the immune responses between the three vaccine age-groups were observed in the zero-inflated model, except for the response to MAP-3 where the Vac8w responded with significantly lower levels. There was no statistically significant effect of the second vaccine booster on IFN- γ responses against vaccine antigens, however, for Vac2w and Vac8w animals the response to SECP-1 was not statistically significant different from controls after only 2 vaccinations.

To investigate if the animal's age had an effect on the IFN- γ producing capacity of blood cells, whole-blood from all vaccinated calves were stimulated with SEB (a bacterial superantigen) at 2 weeks interval throughout the study (Fig. 3) or PHA (Phytohaemagglutinin) at the time of the three vaccinations (data not shown). There was no effect of animal age on the IFN- γ producing capacity.

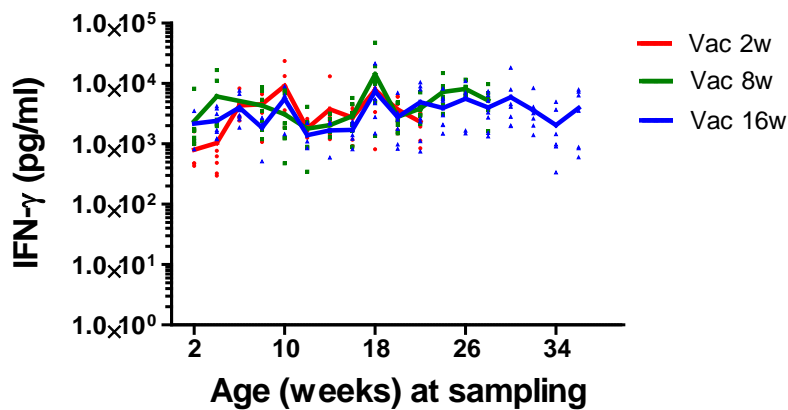


FIG. 3. Secretion of IFN- γ in whole-blood cultures stimulated with SEB from the calves in the vaccinated groups Vac 2w, Vac 8w and Vac 16w. Individual IFN- γ levels are plotted against the age of the calves at the time of blood collection. Line represents mean for each age-group.

Antibody responses to vaccine proteins. Antigen-specific IgG1 levels in serum from immunized calves were measured at first vaccination and 4 weeks after second and third vaccination (Fig. 4). In general, the humoral responses emulate the IFN- γ responses. IgG1 responses were statistically significant to all the vaccine antigens in the vaccinated groups compared to the adjuvant controls. As seen with the IFN- γ responses, the most significant responses were observed against LATP-4, MAP-3, ESAP-2 followed by Ag85B and SECP-1. However no significant differences in the humoral immune responses were observed between the three vaccine groups.

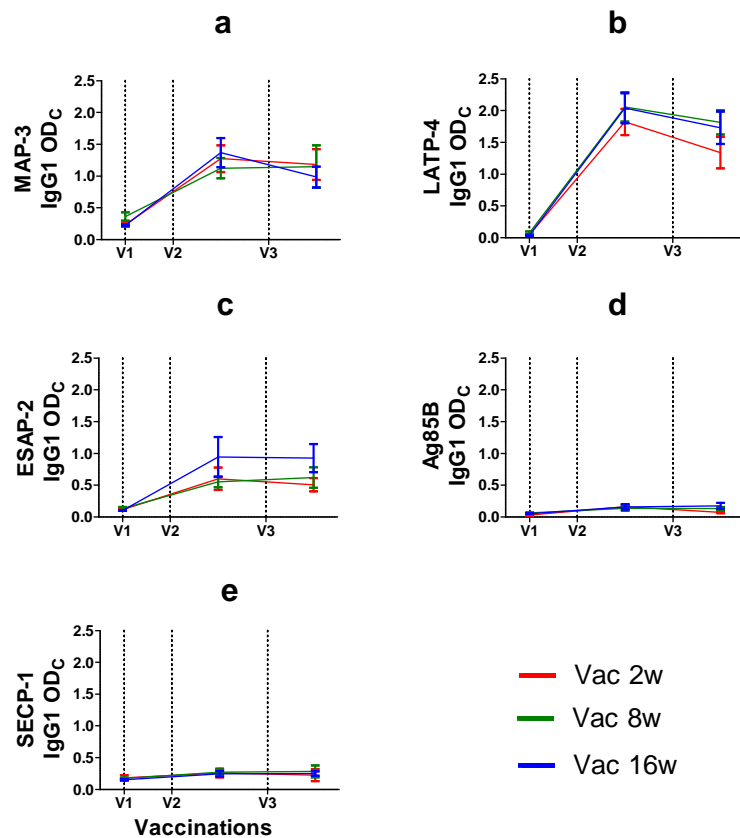


FIG. 4. Seroreactivities to MAP recombinant proteins in calves in groups Vac 2w, Vac 8w and Vac 16w following vaccinations indicated as dotted lines. Antigen-specific IgG1 antibody responses were measured by ELISA and expressed as OD_{cal}. Age at first vaccination (V1) in group Vac 2w, Vac 8w and Vac 16w was 2, 8 and 16 weeks, respectively followed by booster immunizations 4 (V2) and 12 weeks (V3) later. IgG1 antibody production in response to stimulation with MAP recombinant proteins MAP-3 (a), LATP-4 (b), ESAP-2 (c), Ag85B (d), SECP-1 (e). IgG1 levels are expressed as mean values (\pm standard deviation [SD]) for serum OD_{cal} values.

The second vaccination strongly boosted the IgG1 antibody response towards all five vaccine antigens which were statistically strongly significant ($p < 0.0001$) whereas the third vaccination did not increase the IgG1 levels above the level found after the second vaccination. Among the various recombinant antigens used, IgG1 response levels were the highest for LATP-4 (irrespective the coating concentration

for this Ag was only 0.2 g/ml), followed by MAP-3, ESAP-2, Ag85B and SECP-1. None of the pre-vaccination serum samples from any of the calves reacted with any of the MAP recombinant antigens used.

Skin test reactivity of the MAP-vaccinated and control calves. The cross-reactivity with surveillance testing for bovine tuberculosis is a major drawback of available live or killed whole-cell MAP vaccines. Vaccinated calves were therefore evaluated for cross-reactivity using the standard tuberculin skin test six weeks after third vaccination. All 27 calves were found to be negative with the comparative intradermal tuberculin test. The skin test results corroborate with the low IFN- γ levels against PPD_b and PPD_a in the samples collected at that time point (data not shown). Interpretation as single tuberculin test also showed negative reading in all calves except for one with a 5.9 mm reaction to bovine tuberculin and 2.6 mm to avian tuberculin.

DISCUSSION

In this study, we investigated the effect of age on the vaccine induced immune response following subunit vaccination using defined MAP recombinant proteins. Vaccinations in the three groups Vac 2w, Vac 8w and Vac 16w unveiled three immune response profiles. After first vaccination, there was an immediate rise in the IFN- γ levels against vaccine proteins in vaccinated animals compared to the adjuvant control group. In mycobacterial infections including paratuberculosis, CD4 Th1-cell-mediated immunity is essential to keep the infection in check and IFN- γ has a central role (6, 8, 32, 35). Since IFN- γ is a quintessential cytokine in the Th1 response, it is supposedly an immune response variable and a correlate of vaccine induced immune protection (7, 33). In our study, the vaccine proteins did induce a significant IFN- γ response relative to controls indicating that CAF01 adjuvated vaccination induces a measurable antigen-specific cell-mediated immune response in cattle. There was no age-related correlation between vaccinations and the immune response to vaccine antigens which were significant

except for MAP-3 where Vac 8w had significantly lower response levels. One of the possible explanations for the low IFN- γ responses of the calves in the group Vac 8w could be the change in the feeding schedule with milk being replaced with pellets and hay which coincides with the time of first vaccination. Thus a change in the feeding pattern with resulting dietary stress (2, 22) could have modulated the IFN- γ responses following primary vaccination for the group Vac 8w calves and such dietary changes should be taken into account when designing vaccine experiments involving young animals. In addition, the responsiveness of the young cattle in the IFN- γ assay in mycobacterial infections has also been associated with NK cells (24). In our study we didn't look at the IFN- γ production by NK cells and could not associate their role for any non-specific IFN- γ production. Although, one possible scenario could be that NK cells didn't respond to recombinant MAP proteins used in our study.

IFN- γ responses following whole-blood stimulation with SEB and PHA revealed that all the animals in the three vaccine groups produce comparable levels of IFN- γ . Thus, there was no difference in the IFN- γ producing capacity of the animals with age or in response to the vaccinations.

Analysis of the immune responses among the groups considering only two vaccinations revealed a strikingly similar portrait as of three vaccinations, which was statistically significant. Notably, the third vaccination was not able to augment the levels of IFN- γ against all the recombinant vaccine antigens. Thus, it seems here likely that second booster dose for MAP vaccination is trivial at this time point and hence could be omitted.

Among the vaccine antigens used in this study, secreted proteins SECP-1 and Ag85B are expected to be highly immunogenic due to their extracellular environment with high likeliness to encounter the immune cells (25, 31). However, we found that post vaccination immune responses of the animals with

these antigens were weak compared to the other recombinant proteins. Also the antibody responses corroborated this observation. This contrasts with previously published data which showed that recombinant *M. bovis* Ag85B induces strong proliferative and ex-vivo IFN- γ responses following natural and experimental infection in cattle and mice (28). Despite the fact that Ag85B induces an early immune response following infection, whether it plays a major role in the protection against MAP infection when used as a recombinant subunit vaccine is still to be confirmed.

The recombinant MAP antigens encoded by MAP-3, LATP-4 and ESAP-2 induced most significant immune responses with higher IFN- γ levels compared to the secreted proteins. MAP-3, a conserved hypothetical protein the function of which is yet unknown, produced sustained and average higher IFN- γ levels in the animals. LATP-4 is an immunogenic latent protein (heat shock protein) which is known to be expressed during the latent stage of *M. tuberculosis* and may contribute to the control of mycobacterial infection (16). The vaccinated animals in all three groups responded very well to LATP-4 antigen throughout the study period supporting its role as potential candidate in subunit vaccine strategy against MAP infection. Another protein ESAP-2 which is a hypothetical ESAT-6 like protein was found to be comparatively weaker than MAP-3 and LATP-4; however, the responses peaked after first booster in the youngest animal group.

Although vaccinated calves developed strong cell-mediated immune response their humoral responses characterized by IgG1 antibody levels were low but detectable. Post vaccination antibody levels were differentially elevated for LATP-4, MAP-3 and ESAP-2 compared to other recombinant proteins in all three groups. However, no significant differences in the humoral immune response were observed between the vaccine groups. This is in line with recent work suggesting age of the calf and ingestion of colostral antibodies to interfere with the immune capacity of the young calves to produce antigen-specific antibody responses, with little or no effect on cell-mediated immune responses (22,

23). A significant booster effect was noticed following second vaccination for all vaccine antigens. Despite the fact that humoral responses are not considered protective in MAP infection, yet the production of IgG1 antibody in response to recombinant MAP antigens is indicative of the role of vaccine induced IFN- γ in the isotype switching from IgM to IgG (1).

Evaluation of the comparative skin testing results revealed no cross-reaction with either *M. bovis* PPD or *M. avium* PPD tuberculins. Only one calf reacted to bovine PPD tuberculin. This is an important observation considering the fact that the current available whole-cell MAP vaccines show cross-reaction with *M. bovis* PPD and thus pose a problem in the differentiation of the vaccinated and the naturally infected animals. The cross-reactivity of one of the calves to bovine PPD tuberculin in the skin test could not be explained and the same calf reacted poorly to Ag85B in the whole-blood IFN- γ assay performed on sample collected before the skin test. Ag85B is a major component of bovine PPD and the gene encoding the Ag85B protein from MAP shares 85% sequence identity with *M. bovis* Ag85B at the protein level (17). Therefore, at the blood level there was no cross-reactivity between *M. bovis* and MAP for Ag85B.

In summary, we have shown enhanced cell-mediated and humoral immune responses after immunization of calves with recombinant MAP proteins at different ages and that age of vaccination of the calves has some correlation with the vaccine induced immune response to selected MAP proteins. In particular, vaccination of the calves against MAP at the age of weaning could jeopardize the immune response and vaccination of older calves appears to result in more sustained CMI responses. Also, that vaccination followed by a single booster could be sufficient for the generation of ample immune response and thus might circumvent the need for additional boosting of the immune response. We have provided supplementary evidence that vaccination of the calves using selected recombinant MAP proteins and administered as in this study will not result in any side effects and interference with *M.*

bovis diagnosis. Furthermore, it is interesting here in the context of vaccination that MAP-3, LATP-4, and ESAP-2 can elicit immune responses in calves which were statistically significant when administered as recombinant proteins in CAF01 adjuvant. No live MAP experimental challenge was performed in this study as the main purpose was to characterize and compare antigen-specific adaptive immune responses in different age-groups of calves using a cocktail of recombinant MAP proteins.

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ARTICLE II

Increasing the ex vivo antigen-specific IFN- γ production in subpopulations of T cells by anti-CD28, anti-CD49d and recombinant IL-12 costimulation in cattle vaccinated with recombinant proteins from *Mycobacterium avium* subspecies *paratuberculosis*

Aneesh Thakur¹, Ulla Riber¹, William C. Davis², and Gregers Jungersen^{1*}

¹ *Adaptive Immunology and Parasitology, National Veterinary Institute, Technical University of Denmark, Bülowsvej 27, 1870 Frederiksberg C, Denmark*

² *Department of Veterinary Microbiology and Pathology, College of Veterinary Medicine, Washington State University, Pullman, WA, USA*

* Corresponding author: Gregers Jungersen

National Veterinary Institute, Technical University of Denmark

Bülowsvej 27, 1870 Frederiksberg C, Denmark

Phone: +45 35 88 62 34

Fax: +45 35 88 66 01

E-mail: GRJU@vet.dtu.dk

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Increasing the ex vivo antigen-specific IFN- γ production in subpopulations of T cells by anti-CD28, anti-CD49d and recombinant IL-12 costimulation in cattle vaccinated with recombinant proteins from *Mycobacterium avium* subspecies *paratuberculosis*

Aneesh Thakur¹, Ulla Riber¹, William C. Davis², and Gregers Jungersen^{1*}

¹ *Adaptive Immunology and Parasitology, National Veterinary Institute, Technical University of Denmark, Bülowsvej 27, 1870 Frederiksberg C, Denmark*

² *Department of Veterinary Microbiology and Pathology, College of Veterinary Medicine, Washington State University, Pullman, WA, USA*

* Corresponding author. Mailing address: National Veterinary Institute, Technical University of Denmark, Bülowsvej 27, 1870 Frederiksberg C, Denmark. Phone: +45 35 88 62 34. Fax: +45 35 88 66 01. E-mail: GRJU@vet.dtu.dk.

ABSTRACT

T cells which encounter specific antigen (Ag) require additional signals to mount a functional immune response. Here, we demonstrate activation of signal 2, by anti-CD28 mAb (aCD28) and other costimulatory molecules (aCD49d, aCD5), and signal 3, by recombinant IL-12, enhance Ag-specific IFN- γ secretion by CD4, CD8, $\gamma\delta$ T cells and NK cells. Age matched male jersey calves, experimentally infected with *Mycobacterium avium* subsp. *paratuberculosis* (MAP), were vaccinated with a cocktail of recombinant MAP proteins or left unvaccinated. Vaccine induced ex vivo recall responses were measured through Ag-specific IFN- γ production by ELISA and flow cytometry. There was a significant increase in production of IFN- γ by T cell subsets cultured in the presence of Ag and aCD28/aCD49d. The increase was accompanied by an increase in the integrated median fluorescence intensity (iMFI) of activated T cells. Addition of rIL-12 induced a significant additive effect leading to a maximum increase in responder frequency of Ag-specific T cell subsets with a heavy bias towards IFN- γ production by CD4 T cells. We provide the first description of using aCD28/aCD49d costimulation to potentiate an Ag-specific increase in the production of IFN- γ in bovine immunology. The study also shows the degree of signaling in T cells is regulated by the costimulatory environment.

KEYWORDS

Costimulation; IFN- γ ; T cells; Flow cytometry; Cattle

1. Introduction

Johne's disease or paratuberculosis in ruminants is caused by infection with *Mycobacterium avium* subsp. *paratuberculosis* (MAP), and similar to other mycobacterial infections, the relevant immune response is thought to be dominated by CD4+ T cells secreting IFN- γ (Stabel, 2000). In laboratory studies of immune responses to MAP infection or MAP vaccines, it is thus highly relevant to provide optimal conditions for an ex vivo measurement of the recall response of in vivo primed T cells.

Costimulatory signals play a crucial role in all phases of T cell responses including activation, expansion, effector, and memory phases of the response. APCs provide three key signals for the activation of Ag-specific T cells, signal 1 being the TCR cognate recognition of Ag through MHC presentation, signal 2 being costimulation mediated by physical interaction with adhesion and costimulatory molecules on APC with receptors on T cells, and signal 3 mediated by cytokines secreted by APCs, respectively (Kalinski et al., 1999). In ex vivo studies, addition of the target Ag provides the first signal. Addition of monoclonal antibodies specific for ligands/receptors on the T cell surface, mimic the second APC costimulation signals. Addition of recombinantly produced cytokines to the cultures provides the third signals. The CD28/B7 interaction is the prominent and most studied costimulatory signal for T cells (Lenschow et al., 1996). CD80 (B7.1) and CD86 (B7.2) are members of the immunoglobulin superfamily with CD28 as one of their specific ligands. Other costimulatory molecules that belong to the immunoglobulin superfamily include the tumor necrosis factor receptor family, and the integrin family. Integrin molecules are a family of heterodimeric covalently linked α and β glycoproteins. They are involved in the bi-directional signaling in and out of T cells. The family includes VLA-4 (CD49d/CD29). Another costimulatory molecule, CD5 is a type-I transmembrane glycoprotein found essentially on all T cells. Signal 2 costimulatory molecules control T cell proliferation, effector functions and memory development by regulating the intracellular PI3K/PKB/NF- κ B signal transduction pathway (Lenschow et al., 1996). Through this pathway, costimulation controls expansion and proliferation of T cells by sustaining expression of survivin (Song et al., 2005b) and aurora B (Song et al., 2007). Long-term T cell survival and memory generation is mediated

through bcl-2 expression(Song et al., 2005a). IL-12 is a heterodimeric signal 3 cytokine naturally produced by dendritic cells and macrophages which stimulates IFN- γ and TNF production from T cells and NK cells, thereby playing an important role in responses to pathogens like mycobacteria(Xing et al., 2000). Distinct T cell subsets and innate effector cells play a role in immunopathology and protection against mycobacterial infections, including paratuberculosis, through effector cytokine production, chiefly IFN- γ (Ladel et al., 1995; Stabel, 2000). Costimulation of whole blood or PBMC ex vivo with cytokines such as IL-2(Pintaric et al., 2008), IL-12(Jungersen et al., 2012; Mikkelsen et al., 2012) and IL-18(Pintaric et al., 2008; Riber et al., 2011) or monoclonal antibodies blocking inhibitory IL-10 signaling(Mikkelsen et al., 2012) have been investigated extensively in veterinary immunology. However, studies with monoclonal antibodies (mAbs) to costimulatory molecules CD28 and CD49d remain limited with available information mainly from studies in humans and rodents. In this study, we describe the first use of signal 2 costimulation using anti-CD28 (aCD28), anti-CD49d (aCD49d), and anti-CD5 (aCD5) mAbs in cattle, alone or in combination with a costimulatory signal 3 cytokine, recombinant IL-12 (rIL-12), to enhance Ag-specific production of IFN- γ by innate and adaptive T cell subsets. We report on the effect of costimulation on the frequency of T cells responding to MAP Ags in experimentally challenged and vaccinated calves.

2. Materials and Methods

2.1. Animals

Animals used in the present study (N=8, 5 vaccinated and 3 control) were available from a larger post-exposure MAP vaccine trial currently in progress. Male Jersey calves were obtained for the trial (mean age of 2 weeks) from a dairy farm proven to have a true prevalence equal or close to zero by the Danish paratuberculosis surveillance program(Mikkelsen et al., 2011). Animals were housed and raised under appropriate biological containment facilities (BSL-2) in a community pen with straw bedding. All calves were infected with a total of 1×10^9 CFU of a clinical isolate of MAP (Ejlskov 2007) grown in Middlebrook

7H9 medium supplemented with 10% oleic acid-albumin-dextrose complex (Difco, USA) plus 0.05% Tween 80 (Sigma Chemical Co., St. Louis, Mo.) and 2% Mycobactin J (Allied Monitor Inc., USA) and fed with milk replacer (DLG, Denmark) three times over a period of one week from 2 weeks of age. Vaccinated calves ($n = 5$) were immunized by the sub-cutaneous route in the right mid-neck region about 7 cm ahead of the prescapular lymph node at the age of 16 weeks followed by a second vaccination 4 weeks later. All vaccine calves received a recombinant vaccine with 100 μ g each of a fusion protein of four MAP proteins from the ESAT 6 family and a MAP latency protein adsorbed to the adjuvant CAF01 (DDA/TDB; 2500 μ g/500 μ g) for 1 hour at RT before injection (Agger et al., 2008). Selection was based on previous immunogenicity studies in cattle and experimental knowledge from in vitro and in vivo expression studies with *M. tuberculosis* proteins in mice. All Ags were produced in *E. coli* and purified by metal affinity and anion columns as previously described (Mikkelsen et al., 2011). Control calves ($n = 3$) didn't receive any vaccine. Blood samples were collected 8-10 weeks post second vaccination.

All animal experiments were done following approval by and under control of the Danish National Experiments Inspectorate.

2.2. Cell preparation and antigen stimulation

Peripheral blood mononuclear cells (PBMC) were isolated from EDTA cattle blood by Ficoll-Paque PLUS (GE Healthcare Life Sciences) gradient density centrifugation. Cells were then washed twice in RPMI 1640 media (Life Technologies, UK) supplemented with 2% heat-inactivated FCS (Sigma). After washing, cells were resuspended in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin (Gibco, UK), and 100 μ g/ml streptomycin sulphate (Gibco, UK). All cell cultures were performed in 24-well CELLSTAR multi-well plates (Greiner Bio-One, Heidelberg, Germany) containing 5×10^6 cells/well in a volume of 1 ml complete medium with 50 μ l of MAP vaccine Ag cocktail (containing 1

µg of each MAP Ag/ml), 1 µg/ml of the superantigen staphylococcal enterotoxin B (SEB, Sigma-Aldrich) or sterile PBS. Costimulations were performed with combinations of rIL-12 (10 IU/ml (a kind gift from Jayne Hope, The Roslin Institute, University of Edinburgh), anti-bovine CD28 (1 µg/ml; Clone TE1A), anti-bovine CD49d (1 µg/ml; Clone FW3-218), and anti-bovine CD5 (1 µg/ml; Clone CC17; AbD Serotec, UK). The cultures were incubated at 37°C in a humidified 5% CO₂ atmosphere for 18-20 hours. Brefeldin A (10 µg/ml; Sigma-Aldrich) and monensin (2 µM/ml; Sigma) were added for the final 6 hr of culture to block protein secretion for intracellular IFN-γ detection. Following centrifugation of the culture plates, supernatants and cells were collected for IFN-γ analysis by ELISA and flow cytometry, respectively.

2.3. IFN-γ assay

Collected supernatants were stored at -20°C or less until analysis. IFN-γ secretion in supernatants was determined by use of an in-house monoclonal sandwich ELISA as described earlier (Mikkelsen et al., 2009). The levels of IFN-γ (pg/ml) were calculated using linear regression on log-log transformed readings from the two-fold dilution series of a reference SEB stimulated plasma standard with predetermined IFN-γ concentration. The IFN-γ response in PBS cultures was subtracted from IFN-γ response to MAP vaccine Ag cocktail cultures for each sample and defined as the Ag-specific IFN-γ response.

2.4. Flow cytometry for Intracellular cytokine staining (ICS)

After collection of the supernatant, PBMC were harvested following addition of PBS-EDTA (20 mM) with 10 minutes of shaking and pelleted in 96-well FACS plates (BD Biosciences). Surface staining was performed on pelleted cells using one of the four different staining panels (Table 1). All staining was performed at RT for 30 minutes. Cells were stained for surface markers using monoclonal antibodies anti-CD3, anti-CD4, anti-CD8, anti-WC1, and anti-NKp46. Cells were then washed in FACS buffer (PBS containing 0.1 % sodium azide and 1 % Foetal Calf Serum) and subsequently stained with secondary

antibodies as shown in the panel. Cells were then washed in FACS buffer, permeabilized using the Cytofix/Cytoperm kit (BD Pharmingen, USA) according to manufacturer's instructions, and stained for intracellular IFN- γ for 30 minutes at RT. Cells were subsequently washed in perm wash buffer (BD Pharmingen, USA), resuspended in FACS flow, and analyzed using an eight-color BD FACSCanto II flow cytometer (BD Biosciences, USA) with a High Throughput Sampler (HTS) 96-well plates. Data analysis was performed using BD FACSDiva software vs. 6.1.2.

Table 1: Flow cytometry staining panels

Primary staining					Secondary staining	
Panel	Monoclonal antibodies	Isotype	Clone	Source	Secondary antibodies	Fluorochrome
1	anti-CD3	IgG1	MM1A	VMRD	anti mouse IgG1	FITC
	anti-CD4	IgG2a	IL-A11	VMRD	anti mouse IgG2a	RPE
	anti-IFN γ	IgG1	CC302	AbD Serotec	anti mouse IgG1	AF647
2	anti-CD3	IgG1	MM1A	VMRD	anti mouse IgG1	FITC
	anti-CD8	IgG2a	CC63	AbD Serotec	anti mouse IgG2a	RPE
	anti-IFN γ	IgG1	CC302	AbD Serotec	anti mouse IgG1	AF647
3	anti-CD3	IgG1	MM1A	VMRD	anti mouse IgG1	FITC
	anti-WC1	IgG2a	CC15	AbD Serotec	anti mouse IgG2a	RPE
	anti-IFN γ	IgG1	CC302	AbD Serotec	anti mouse IgG1	AF647
4	anti-NKp46	IgG1	AKS1	Anne K. Storset, Norway	anti mouse IgG1	FITC
	anti-CD8	IgG2a	CC63	AbD Serotec	anti mouse IgG2a	RPE
	anti-IFN γ	IgG1	CC302	AbD Serotec	anti mouse IgG1	AF647
5	Isotype control	IgG1	-	Southern Biotech	anti mouse IgG1	FITC
		IgG2a	-	Southern Biotech	anti mouse IgG2a	RPE
		IgG1	-	AbD Serotec	anti mouse IgG1	AF647

Integrated median fluorescence intensity (iMFI) was calculated by multiplying the relative frequency of cells expressing IFN- γ with the MFI of IFN- γ signal. This provides a quantitative assessment of the magnitude as well as the quality of the cytokine response for different cell subsets (Seder et al., 2008). MAP vaccine Ag-specific iMFI was calculated by deducting iMFI responses to PBS cultures from those of MAP vaccine Ag cocktail (Cordes et al., 2012).

2.5. Statistical analysis

Comparison of MAP vaccine Ag-specific IFN- γ release and IFN- γ iMFI responses between different costimulations in vaccinated calves was performed using non-parametric Friedman test and Dunn's multiple comparison test. Differences between the magnitude of responses in signal 2 and signal 3 costimulations and their interaction effect were determined using repeated measures ANOVA with Bonferroni post analysis test. Comparison of immune responses between vaccinated and control calves were done using a non-parametric two-tailed Mann-Whitney test. Statistical analysis was performed using GraphPad Prism software vs. 5 (GraphPad Software Inc., USA).

3. Results and Discussion

Production of IFN- γ by distinct T cell lineages(Coussens, 2001; Kaufmann, 2002) and innate effector cells(Ladel et al., 1995) plays an important role in the pathogenesis as well as resistance to mycobacterial infections. In MAP infection, the inflammatory environment in the small intestine especially the jejunum, distal ileum, and associated lymph nodes provides necessary costimulatory signals(Stabel, 2000; Coussens, 2001) that might be missing on evaluating the IFN- γ responses in ex vivo PBMC assays. This might lead to detection of only low activation threshold T cells while missing T cell populations with a higher threshold of activation. The addition of costimulatory molecules compensates for this deficit and enhances the sensitivity of PBMC, as well as whole-blood based assays(Waldrop et al., 1998), by bringing both qualitative and quantitative changes leading to lower activation threshold and an increase in T cell activity. Here, we demonstrate an increase in the amount of IFN- γ production by Ag-specific T cells cultured with IL-12 and mAbs to one or more costimulatory molecules. The data presented in this communication is based on a small subset of animals of a larger trial as described earlier. However, the results from this study enabled us to select the best combination of costimulatory molecules for the rest of the vaccine trial.

3.1. Effects of addition of costimulatory molecules on IFN- γ release by PBMCs

PBMCs from the vaccinated and control calves were stimulated with antigen alone or in combination with IL-12 and costimulatory mAbs. IFN- γ levels were measured in the culture supernatant. Comparison of the different culture conditions revealed significant differences in the capacity of combinations of IL-12 and costimulatory mAbs to enhance the production of IFN- γ (Fig. 1).

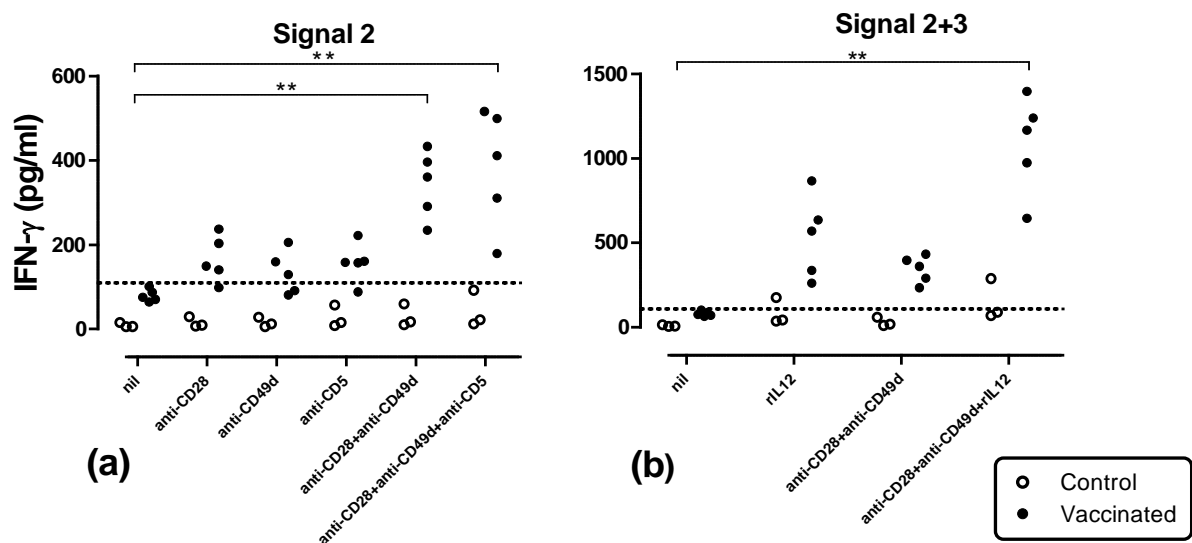


Figure 1. Antigen-specific IFN- γ responses in 18 hrs PBMC cultures with signal 2 and 3 costimulatory molecules added. (a) Levels of IFN- γ released from PBMC cultures stimulated with recombinant vaccine proteins (Ag) costimulated with aCD28, aCD49d, aCD5, aCD28/aCD49d, and aCD5/ aCD28/aCD49d. (b) Levels of IFN- γ released from PBMCs cultured with Ag and costimulated with rIL-12, aCD28/aCD49d, and rIL-12/aCD28/aCD49d. IFN- γ levels are expressed as mean values (\pm standard deviation [SD]) for plasma concentrations (pg/ml). Dotted line is the mean+2SD of IFN- γ production levels of vaccinated animals with nil costimulation (only Ag). Significant differences between nil costimulation (Ag stimulated) and signal 2 or 3 costimulation were determined by non-parametric Friedman test and Dunn's multiple comparison test analysis (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Among the combinations used in this study, both aCD28/aCD49d and aCD5/aCD28/aCD49d were found to significantly ($p<0.01$) elevate the MAP vaccine Ag-specific IFN- γ production compared to control animals (Fig. 1a). aCD5/aCD28/aCD49d increased the non-specific background in one control animal. In terms of interaction effect, there was a significant interaction ($p<0.05$) between aCD28 and aCD49d mAb.

Relative to costimulation with mAbs alone, a further increase in the MAP vaccine Ag-specific IFN- γ production was obtained by addition of rIL-12 and the combination of rIL-12 with aCD28/aCD49d further increased IFN- γ release compared to rIL-12 alone (Fig. 1b). This combination, however, also led to an increase in the background IFN- γ release in control animals with very high levels in one of the controls. In contrast, aCD28/aCD49d didn't increase the Ag-specific IFN- γ production in the control animals.

3.2. Costimulation with Signal 2 molecules increase iMFI of Ag-specific IFN- γ producing T cell subsets

To estimate which subset of T cell populations (CD4+, CD8+, $\gamma\delta$, or NK cells) might be responsible for the IFN- γ production with signal 2 costimulatory mAbs, we used flow cytometry for measuring Ag-specific IFN- γ iMFI in PBMC culture from vaccinated and control calves (Supplemental figure 1). The frequency of effector T cells and NK cells responding to MAP vaccine Ag cocktail with production of IFN- γ were found to increase in the presence of aCD28, aCD49d, or aCD5 mAb, as well as their combinations (Fig. 2).

Costimulation with aCD28 was found to enhance more IFN- γ iMFI as compared to aCD49d or aCD5 mAb alone in all cell subsets. Maximum increase in the IFN- γ iMFI with low non-specific background signal was observed with aCD28/aCD49d combined. A significant effect of interaction ($p<0.05$) was observed with this combination in all T cell subsets except CD8+NKp46+ cells (Fig. 2d). However, addition of aCD5 mAb was found to increase the background signal in the control animals either alone or in combination with aCD28/aCD49d in all IFN- γ producing subsets with no significant interaction effect. No significant

change in the distribution of IFN- γ producing cells among the T cell subsets was observed after addition of aCD28/aCD49d.

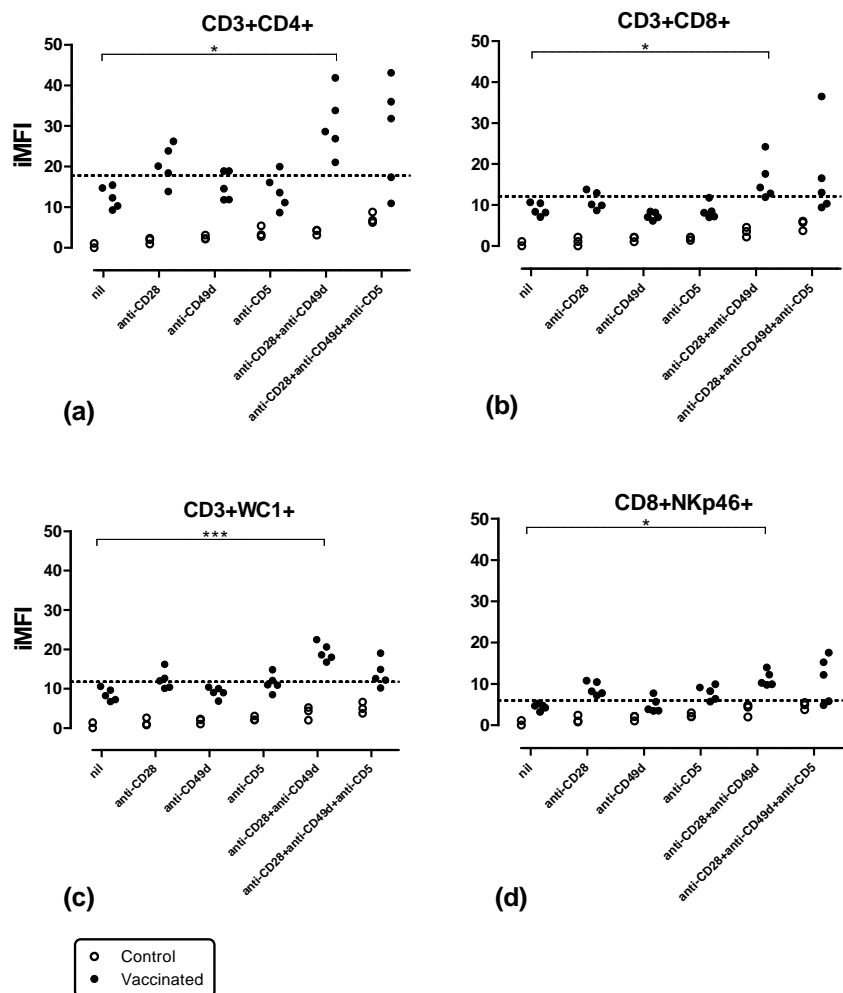


Figure 2. Antigen-specific IFN- γ iMFI of T cell subsets in PBMC cultures from vaccinated or control animals costimulated with mAbs aCD28, aCD49d and aCD5. Antigen-specific iMFI was calculated as iMFI from cultures with Ag – iMFI from cultures with PBS. (a) iMFI of CD3+CD4+ subsets, (b) iMFI of CD3+CD8+ subsets, (c) iMFI of CD3+WC1+ subsets, (d) iMFI of CD8+NKp46+ subsets. Dotted line is the mean+2SD of IFN- γ iMFI of vaccinated animals with nil costimulation (only Ag). Significant differences between nil costimulation (Ag stimulated) and signal 2 costimulation were determined by non-parametric Friedman test and Dunn's multiple comparison test analysis (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Summarized, these data show there is an increase in the Ag-specific IFN- γ iMFI after addition of signal 2 or other costimulatory molecules and their combinations with cross-linking of CD28 molecules as the most important costimulation as compared to aCD49d or aCD5 mAb alone and with an additive effect of aCD28/aCD49d. Possibilities of nonspecific or cross-reactive cytokine responses were not apparent as these mAbs had no stimulatory effect in the PBS stimulated samples (data not shown). This suggests that there exists a spectrum of T cell activation thresholds, with some requiring only one strong signal while others requiring a combination of signals. It has been reported that signaling induced by CD28 cross-linking primarily affects IL-2 production which further plays a role in T cell priming and augments T_h1 (IFN- γ) and T_h2 type (IL-4, IL-5, and IL-10) cytokine production (Seder et al., 1994). Other studies reported that addition of aCD28 didn't lead to significantly increased per cell IFN- γ production, but rather an increased frequency of cytokine producing T cells (Waldrop et al., 1998). However, aCD28/aCD49d has been found to potentiate the low threshold T cells in many low infection disease models and vaccine experiments (Horton et al., 2007; Lindenstrom et al., 2009; Geluk et al., 2010).

3.3. Signal 3 costimulation induce maximum iMFI of Ag-specific IFN- γ producing T cell subsets

To further explore the potentiation of IFN- γ levels and contribution of distinct T cell subsets, we analyzed the iMFI of Ag-specific IFN- γ producing T cells after addition of signal 3 cytokine rIL-12 and its combination with aCD28/aCD49d. In all lymphocyte subsets, there was a significant effect of interaction ($p < 0.01$) between rIL-12 and aCD28/aCD49d that was most prominent in case of CD3+CD8+ and CD8+NKp46+ populations ($p < 0.001$). By screening IFN- γ iMFI of the CD3+CD4+ T cells with rIL-12 and its combinations, it was observed that signal 3 cytokine alone (~40 fold) and together with aCD28/aCD49d (~56 fold), significantly increase the Ag-specific IFN- γ iMFI in vaccinated compared to control animals (Fig. 3a). This increase was 14 and 26 fold in CD3+CD8+ T cells (Fig. 3b) and 2.5 and 21 fold in CD3+WC1+ T cells, respectively (Fig. 3c). In the CD8+NKp46+ subset an increase of about 4 fold was observed with both signal 3 as well as signal 2 and 3 costimulatory molecules (Fig. 3d).

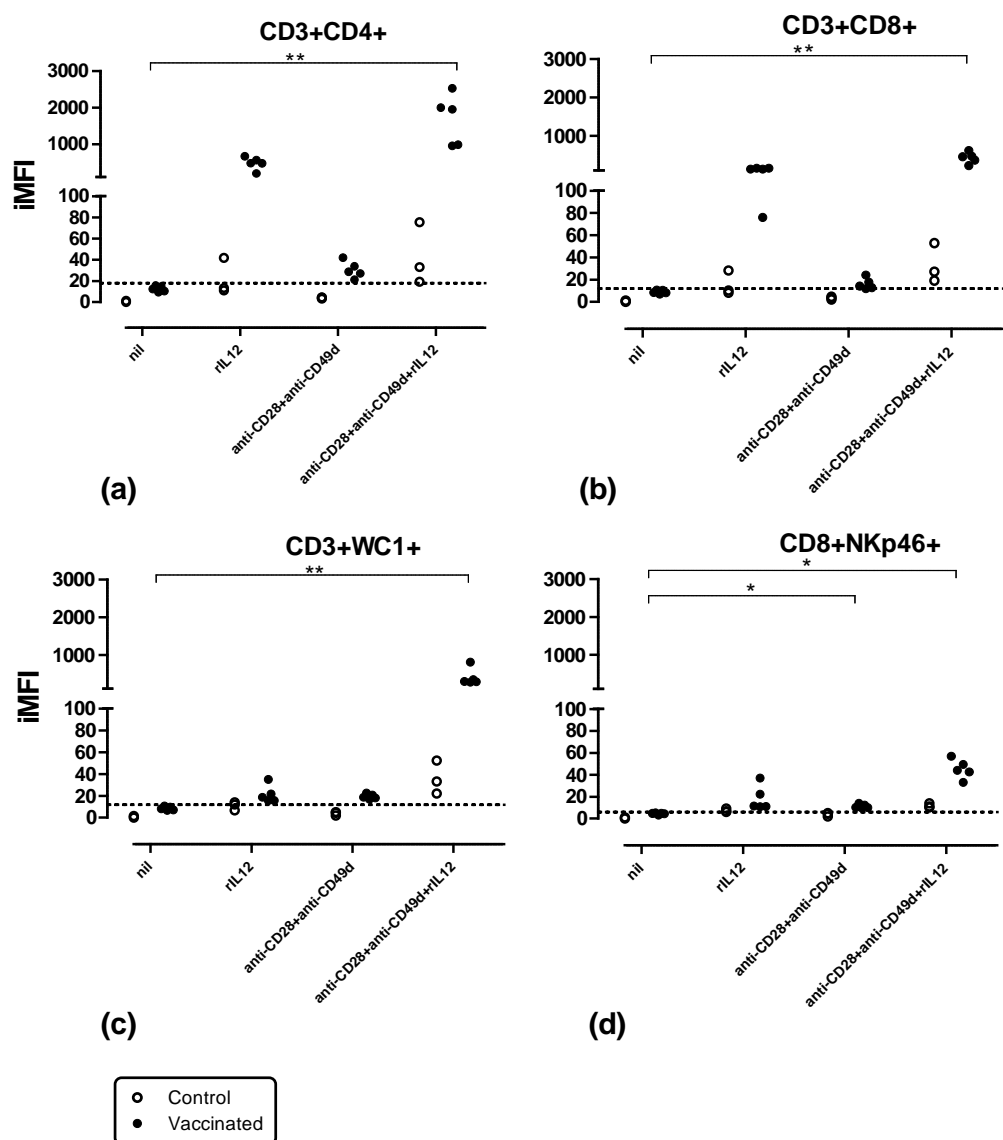


Figure 3. Antigen-specific IFN- γ iMFI of T cell subsets in PBMC cultures from vaccinated or control animals costimulated with signal 3 (rIL-12) and signal 2 (aCD28/aCD49d) costimulatory molecules (a) iMFI of CD3+CD4+ subsets (b) iMFI of CD3+CD8+ subsets (c) iMFI of CD3+WC1+ subsets (d) iMFI of CD8+NKp46+ subsets. Dotted line is the mean+2SD of IFN- γ iMFI of vaccinated animals with nil costimulation (only Ag). Significant differences between nil costimulation (Ag stimulated) and signal 3 costimulation were determined by non-parametric Friedman test and Dunn's multiple comparison test analysis (* $p<0.05$, ** $p<0.01$, *** $p<0.001$).

260 The strong potentiation of IFN- γ with rIL-12, however, also increased the background in the control
 261 animals that was absent in case of aCD28/aCD49d costimulation. While the profile of IFN- γ producing
 262 cells after aCD28/sCD49d costimulation still reflected the profile without costimulation, the marked
 263 increase in the IFN- γ production of CD3+CD4+ T cells after addition of rIL-12, with or without
 264 aCD28/aCD49d, skewed the profile of IFN- γ producing cells towards a CD3+CD4+ T cell dominance.
 265
 266 Signal 3 inflammatory cytokines such as IL-12, IFN- γ , and type I IFNs regulate multiple aspects of the T
 267 cell response including expansion, differentiation and effector functions(Kaech and Ahmed, 2001).
 268 However, the signal 3 inflammatory signature in vivo is pathogen-specific as different types of
 269 pathogens are likely to induce different inflammatory cytokine environments. IL-12 stimulates the
 270 production of IFN- γ by the activation of transcription factor STAT4(van, V et al., 2003) and has been used
 271 as a costimulatory molecule(Valenzuela et al., 2002; Geluk et al., 2010) as well as an immunostimulatory
 272 agent or adjuvant in vaccination protocols(Afonso et al., 1994). There has been evidence of IL-12
 273 mediated augmentation of IFN- γ secretion by $\gamma\delta$ T cells and NK cells(Skeen and Ziegler, 1995). IL-12 has
 274 earlier been shown to significantly increase the IFN- γ production with a corresponding increase in the
 275 percentages of single IFN- γ +CD3+CD4+ T cells(Geluk et al., 2010). Supplemental exogenous rIL-12 has
 276 been demonstrated to show a dose-dependent capacity to synergistically promote IFN- γ production in
 277 both in vitro and in vivo conditions after BCG vaccination(O'Donnell et al., 1999).
 278
 279 Taken together, our data show that maximum Ag-specific IFN- γ production can be measured with a
 280 combined signal 2 and 3 costimulation in the form of aCD28/aCD49d and rIL-12. The data also show that
 281 individual subsets and cells differ in their costimulatory requirements for activation as well as IFN- γ
 282 production. With the strong shift towards a Th1 type response following exogenous IL-12
 283 supplementation, this costimulation is likely to introduce a bias in the ex vivo read-out and should
 284 preferentially be used to support an Ag-specific IFN- γ production e.g. for diagnostic purposes. In other

ex vivo applications where costimulation is intended to enhance a more complete picture of the ongoing Ag-specific immune responses, e.g. to characterize CMI responses in response to infection or vaccination, the powerful signal 3 costimulation should not be used. In this place a signal 2 costimulation with aCD28/aCD49d appears to promote a less biased read-out.

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ARTICLE III

Immune Markers and Correlates of Protection for Vaccine Induced Immune Responses

Aneesh Thakur, Lasse E. Pedersen and Gregers Jungersen*

*Adaptive Immunology and Parasitology, National Veterinary Institute, Technical University of Denmark,
Bülowsvej 27, 1870 Frederiksberg C, Denmark*

* Corresponding author: Gregers Jungersen

National Veterinary Institute, Technical University of Denmark

Bülowsvej 27, 1870 Frederiksberg C, Denmark

Phone: +45 35 88 62 34

Fax: +45 35 88 66 01

E-mail: GRJU@vet.dtu.dk

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Review

Immune markers and correlates of protection for vaccine induced immune responses

Aneesh Thakur, Lasse E. Pedersen, Gregers Jungersen*

Adaptive Immunology and Parasitology, National Veterinary Institute, Technical University of Denmark, Bülowsvej 27, 1870 Frederiksberg C, Denmark

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ABSTRACT

Vaccines have been a major innovation in the history of mankind and still have the potential to address the challenges posed by chronic intracellular infections including tuberculosis, HIV and malaria which are leading causes of high morbidity and mortality across the world. Markers of an appropriate humoral response currently remain the best validated correlates of protective immunity after vaccination. Despite advancements in the field of immunology over the past few decades currently there are, however, no sufficiently validated immune correlates of vaccine induced protection against chronic infections in neither human nor veterinary medicine. Technological and conceptual advancements within cell-mediated immunology have led to a number of new immunological read-outs with the potential to emerge as correlates of vaccine induced protection. For T_H1 type responses, antigen-specific production of interferon-gamma (IFN- γ) has been promoted as a quantitative marker of protective cell-mediated immune responses over the past couple of decades. More recently, however, evidence from several infections has pointed towards the quality of the immune response, measured through increased levels of antigen-specific polyfunctional T cells capable of producing a triad of relevant cytokines, as a better correlate of sustained protective immunity against this type of infections. Also the possibilities to measure antigen-specific cytotoxic T cells (CTL) during infection or in response to vaccination, through recombinant major histocompatibility complex (MHC) class I tetramers loaded with relevant peptides, has opened a new vista to include CTL responses in the evaluation of protective immune responses. Here, we review different immune markers and new candidates for correlates of a protective vaccine induced immune response against chronic infections and how successful they have been in defining the protective immunity in human and veterinary medicine.

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1. Introduction

The development of novel vaccines is a lengthy process and requires scientifically proven basic research in animal models, safety testing and prolonged clinical testing in humans before reaching the licensure phase. In cases of seasonal diseases and pandemics, newly emerging diseases and chronic infections with long stages of latency, clinical trials are not always feasible. In such cases, where measuring the immune response from the clinical endpoint is impossible, the rational development of vaccines, and in some cases even their approval, will rely on demonstration of immunological correlates of protection after vaccination. In addition, validated correlates of protection may be used to ensure vaccine potency and efficacy in batch-to-batch control and serial validation. According to the Food and Drug Administration (FDA), a correlate of protection is a laboratory parameter which

is associated with protection from the occurrence of clinical disease as shown after sufficient and controlled trials [1]. A correlate of protection can thus only be identified following statistical analysis within a large group of vaccinated or infected individuals in which some of the individuals are protected and others are not. Recently, it has been proposed to subdivide correlates of protection into (i) mechanistic correlates of protection, when the measured immune parameter is causally responsible for the observed protection, and (ii) non-mechanistic correlates of protection, when the measured parameter is significantly correlated with protection, but not a mechanistic causal agent of the protection [2]. In many cases descriptions of immune responses that reflect likely biological functionality are provided even though they have not been shown to statistically correlate with protection. Such measures should be labeled as relevant immune markers and not confused with correlates [3]. Similarly, it will not always be feasible to use development of (or protection against) the actual clinical disease to evaluate the efficacy of a vaccine, in which case the use of surrogate clinical endpoints become necessary. Surrogates are thus laboratory or physical signs (correlates) that are used in clinical trials as a

* Corresponding author. Tel.: +45 35 88 62 34; fax: +45 35 88 66 01.

E-mail address: GRJU@vet.dtu.dk (G. Jungersen).

substitute for a clinically meaningful endpoint. Surrogates may be used to predict protection or meaningful clinical benefit when an absolute immune correlate of protection is unknown [3,4].

A number of immunological parameters have been investigated as potential immune correlates of protection (Table 1). These can be broadly categorized into cell-mediated and humoral immune correlates and must reflect induction of the type of immunological response that is required for protective efficacy against the relevant pathogen (Fig. 1). The majority of vaccines have been developed empirically, with little or no understanding of a known mechanism of immune protection, and the success behind these vaccines is mostly related to induction of strong humoral immune responses by eliciting long lived plasma cells (Table 2). However, for chronic intracellular infections caused by e.g. *Mycobacteria*, *Chlamydia*, *Apicomplexa* parasites and others, the vaccinology research and development has been challenged by agents that are not easily controlled by antibodies [5]. In such diseases, it is the cell-mediated immune (CMI) response of the adaptive immune system that is central to the mediation of protection. Since empirical vaccine development so far has proven unsuccessful for these infections, it can be argued that a more rational approach of vaccine development is needed to activate an effective CMI response [6]. Such an approach should be based on an understanding of the molecular basis of microbial pathogenicity, host–pathogen interactions and the nature of the protective T cell responses for the individual infection. Irrespective of whether vaccine development against chronic intracellular infections will be most successful through an empirical vaccinology approach or applied immunological research, it is imperative to establish methods that assess the magnitude and quality of particular immune responses after vaccination. Such methods can be used to guide the vaccine development and could also be developed into potency tests.

2. Immune correlates of antibody-mediated immunity

In late years of the 20th century it was largely believed that immunity to intracellular pathogens is conferred by a CMI response (T_H1) while immunity to extracellular pathogens rely on antibody-mediated mechanisms (T_H2). However, this notion of immunological duality has been challenged by the fact that antibody-mediated immunity confers protection in most viral vaccines despite their obligate intracellular life. There is convincing evidence that intracellular pathogens elicit a mixed immune response characterized by both T_H1 and T_H2 type response. These mixed protective responses have been very well demonstrated for

Chlamydia, *Plasmodium*, *Schistosoma* and *Cryptococcus neoformans* infections [7–9].

Almost all available licensed vaccines elicit a robust antibody response that correlates with the level of protection extended by the vaccine (Table 2) [10,11]. This is due to the fact that most pathogens at some point in the pathogenesis are susceptible to antibody neutralization such as during replication on mucosal surfaces, in an extracellular state after passage through the bloodstream or in a transient extracellular phase. However, recent findings have also revealed a significant immunoregulatory effect of antibodies on T-cell immunity against *Mycobacteria*, *Chlamydia* and others [12]. Specific antibody isotypes modulate T_H1 activation via Fc receptors (FcR) by facilitating a rapid uptake, processing and presentation of pathogen-derived antigen (Ag) for a strong T-cell response [13]. Thus a protective T-cell memory response appears to require an effective primary humoral immune response [7,12]. Many characteristics of antibodies such as titer, phenotype, function, specificity and location have been investigated in a number of immunological assays as potential correlates of immune protection in various vaccine trials (Table 1). Most of these assays have been used for quantifying and defining the protective concentrations and thresholds of the antibodies against infections.

2.1. Neutralizing antibody titer

Persisting antibody following vaccination has been recognized as a first line of host defense against infection and in the past regarded as a hallmark of an effective vaccine [14,15]. Moreover, most of the successful commercially available vaccines have the ability to induce strong antibody responses which are protective. Several commercial vaccines induce defined serum antibody titers as measured by binding using an enzyme-linked immunosorbent assay (ELISA) as well as haemagglutination and neutralization assays which serve as correlates or markers of protection [15]. Smallpox and yellow fever vaccines which are both live attenuated vaccine are classical examples. Both have been developed at a time when no immunological techniques were available to define the immunological correlates. However, vaccine experiments around 1970s confirm the neutralizing antibody titers as the key players of protective immunity with 1:20–1:32 for smallpox [16,17] and 0.7 LNI (log neutralizing index) for yellow fever [18]. Likewise, serum neutralizing antibody titers for a variety of intracellular pathogens particularly the viruses have been defined (Table 2). However, defined threshold levels of protective antibody titers for intracellular bacterial infections remains elusive. The antibody response is crucial for preventing many intracellular bacterial and

Table 1
Immunological parameters investigated as potential correlates of protection.

Parameter	Response/mechanism	Correlate of protection	References
Humoral			
Neutralizing antibodies	Humoral	Correlate	[17,220–223]
Serum IgG	Humoral	Correlate	[220,224–228]
Mucosal IgG, IgA	Humoral	Correlate	[38,229,230]
Cell-mediated			
IFN- γ	T_H1	Inconsistent data	[84–86,98,231,232]
TNF- α	T_H1	ND	[74,110,112]
IL-2	T_H1	ND	[75,112,114]
IL-17	T_H17	ND	[116,119,233]
IL-1, 4, 10	T_H2	ND	
IFN- γ , TNF- α , IL-2	Polyfunctional T cells	Inconsistent data	[79,112,168,169]
MHC class I and II tetramer-specific T cells	MHC restriction	ND	[127,128,134,141,143]
CCR7 ^{hi} , CD45RA ⁺ , CD62 ^{hi} , CD44 ⁺ , CD127 ^{hi} , CD27 ⁺ , CD28 ⁺	T_{CM} : IL-2, IFN- γ ^{low} , IL-4 ^{low} , IL-17A ^{low}	Inconsistent data	[65,155,156,158]
CCR7 ^{low} , CD45RA ⁺ , CD62 ^{low} , CD44 ⁺ , CD127 ^{low} , CD27 ⁺ , CD28 ⁺	T_{EM} : IFN- γ , IL-2, IL-4, IL-17A	Inconsistent data	[74,161,163,164]
CD27 ⁺ , FCRL4 ⁺	Memory B cells, humoral	Surrogate	[56,59]
$\gamma\delta$ TCR ⁺ , CD3 ⁺	$\gamma\delta$ T cells: IFN- γ , IL-17A, IL-17F, IL-22	ND	[186–189]
CD3 ^{low} CD56 ⁺ NKT cells	IFN- γ , IL-17A, IL-4	ND	[175,176]

ND, not determined.

Table 2

Thresholds of vaccine induced correlates and surrogates of protection (FDA licensed vaccines).

Vaccine	Trade name (s)	Vaccine type	Test	Threshold of protection	Serum IgG	Mucosal IgG	Mucosal IgA	T cells	References
Hepatitis A	Havrix, VAQTA	Inactivated	ELISA	10 mIU/ml	++				[224,234]
Hepatitis B	Recombivax HB, Engerix-B	Recombinant	ELISA	10 mIU/ml	++				[225]
Hib polysaccharide (Hib PS)	Hib vaccine	Polysaccharide (PS)	ELISA	1 µg/ml	++	+			[235]
Hib conjugate	PedvaxHIB, Pentacel	PS-protein Conjugate	ELISA	0.15 µg/ml	++	++			[236]
Human papillomavirus	Gardasil, Cervarix	Virus-like particles	ELISA	ND	++	++			[230]
Influenza	No trade name	Inactivated/live attenuated/(intranasal)	HI	1:40 titer	++	+		+ (CD8+) (intranasal)	[226]
Japanese encephalitis	JE-vax, Ixiaro	Inactivated/live attenuated	Neutralization	1:10 titer	++				[220]
Measles	MMRII, Attenuvax	Live attenuated	Microneutralization	120–200 mIU/ml	++			+ (CD8+)	[221,222]
Meningococcal	Menactra	Polysaccharide	Bactericidal	1/4	++	+			[228]
Mumps	MMRII, Mumpsvax	Live attenuated	Neutralization	ND	++				[223]
Polio	Poliovax, IPOL	Live attenuated/inactivated	Neutralization	1:4–1:8 titer	++	++	++		[38]
Rabies	Imovax, RabAvert	Inactivated	Neutralization	0.5 IU/ml	++				[237]
Rotavirus	ROTARIX, Rotateq	Live attenuated	Serum IgA	ND			++		[229]
Rubella	MMRII, Meruvax II	Live attenuated	Immunoprecipitation	10–15 mIU/ml	++				[227,238]
Smallpox	ACAM2000	Live attenuated	Neutralization	1:20–1:32 titer	++				[16,17]
Tuberculosis	BCG	Live attenuated	IFN-γ, Polyfunctional T cells	ND				++ (CD4+)	[231]
Varicella	Varivax	Live attenuated	FAMA, gp ELISA	1:64 titer, 5 IU/ml	++			+ (CD4+)	[239,240]
Varicella zoster	Zostavax	Live attenuated	CD4+ T cell proliferation	ND	++				[241]
Yellow fever	YF-Vax	Live attenuated	Neutralization	0.7 LNI	++				[18]

Adapted from Plotkin (2008, 2010) with modifications.

ND, not defined.

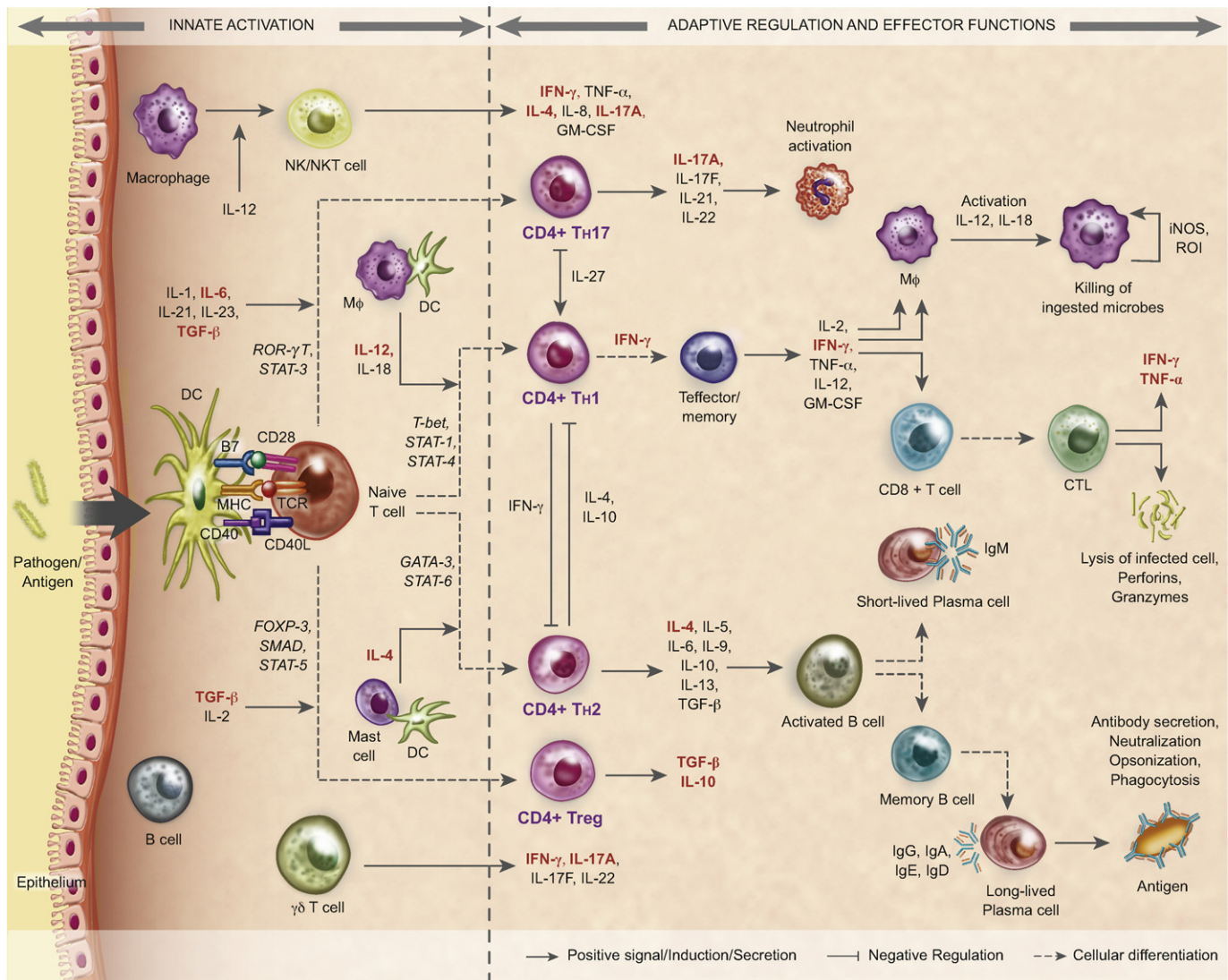


Fig. 1. Schematic representation of the different stages involved in the induction and regulation of cell-mediated and humoral immune response against pathogens. **Stage 1: Innate activation** – Pathogens or antigens can be taken up by antigen presenting cells (APC), namely macrophages, dendritic cells (DCs) or B-cells once they breach the epithelial barrier. Antigens are presented to the naive T-cells by the activated DCs through MHC–TCR interaction (signal 1). Interaction of adhesion and costimulatory molecules on APC such as CD80 (B7.1) and CD86 (B7.2), with CD28 on the surface of T cells and CD40 on B cells with CD40L on Th2 cells (signal 2) leads to activation and expansion of antigen-specific T-cells. Cytokine production by APCs (signal 3) and innate activation involving interaction of pathogen-associated molecular patterns (PAMPs) with Toll-like receptors (TLRs), cytokines milieu (IL-1, -2, -4, -6, -12, -18, -21, -23, TGF-β), transcription factors (T-bet, STAT-1, -3, -4, -5, -6, GATA-3, ROR-γT, FoxP-3, SMAD) leads to activation and differentiation of naive T cells into either, Th1, Th2, Th17 or Treg cell lineage. **Stage 2: Adaptive regulation and effector functions** – This stage is characterized by the cell-mediated immunity through Th1 cells and humoral immunity through Th2 cells. Th1 cells activate macrophages and cytotoxic lymphocytes (CTL) through secretion of cytokines (IFN-γ, IL-2, -12, -18, TNF-α, GM-CSF) enabling them to destroy pathogens or induce apoptosis in infected cells. Th2 cells activate B-cells through production of cytokines IL-4, -5, -6, -10, -13, TGF-β and induce B-cell differentiation, antibody class-switching and affinity maturation. Th17 and Treg cells participate in neutrophil activation and immune regulation by producing cytokines IL-17A, -F, -21, -22 and IL-10, TGF-β, respectively. Th1 effector functions are characterized by killing of the ingested microbes through maximized killing efficacy of macrophages by producing inducible nitric oxide synthase (iNOS) and reactive oxygen intermediates (ROI) and lysis of infected cells by CTL through perforins and granzymes. Th2 effector functions represent increased neutralizing antibody production, opsonization and phagocytosis. Activated T cells differentiate into memory phenotypes-effector memory (T_{EM}) homing to peripheral tissues and central memory (T_{CM}) homing to secondary lymphoid organs. While activated B cells differentiate into short-lived (secrete IgM) and long-lived plasma cells (secrete IgG, IgA, IgE, IgD). γδ T cells and NK/NKT cells serve as a bridge connecting the stages 1 and 2. γδ T cells and NK/NKT cells modulate the immune response by producing both pro and anti-inflammatory cytokines (IFN-γ, IL-17A, -17F, -21, -22).

viral infections and may also contribute to resolution of infection. Antibodies can neutralize viral infectivity in a number of ways, they may interfere with virion binding to receptors, block virus uptake into cells, prevent uncoating of the viral genome in endosomes, or cause aggregation of virus particles. Also many enveloped viruses are lysed when antiviral antibodies and serum complement disrupt membranes. Many studies have investigated the role of neutralizing antibodies for protection against non-bacterial non-viral intracellular pathogens. Neutralizing species-specific or serovar-specific antibodies are produced in response to *Chlamydia* infection in humans and in some animal species [19]. High levels

of *Chlamydia*-specific neutralizing antibody titers (IgG, IgA) were reported in various vaccine experiments with no [20], partial [21] and optimal protective immunity [22]. In malaria, antibodies with neutralizing properties have been reported to be induced during infection and provide support for their potential importance in the development of vaccines and immunotherapy [23,24]. Neutralizing antibody responses of the subclasses IgG1 and IgG3 have been associated with protection from clinical malaria following merozoite vaccination [25]. Although *Mycobacteria* are prototypical for a CMI response, neutralizing antibodies of the subclass IgG and IgA have been found to prolong survival against tuberculosis in

various vaccine experiments through Ag clearance and preventing dissemination of the bacilli [26,27].

2.2. Antibody dependent cell-mediated cytotoxicity (ADCC)

ADCC is one of the mechanisms through which the antibodies can act to limit and contain infection. Classical ADCC is mediated by natural killer cells with membrane bound Fc receptors (FcγRIII or CD16) which bind to antibody-coated (IgG1 or IgG3) microbes and execute killing through various modalities, perforin, granzymes, reactive oxygen intermediates and cytokines. Neutrophils, eosinophils and macrophages can also mediate ADCC through FcγRII or FcγRIII, FcεRI, and FcγRII, respectively. ADCC eliciting properties of antibodies or effector cells can be measured through various labeling assays such as chromium-51 [Cr^{51}] release assay, europium [Eu] release assay and sulfur-35 [S^{35}] release assay. A coupled bioluminescence based non-radiometric GAPDH (glyceraldehyde-3-phosphate dehydrogenase) enzyme release assay is now widely used for ADCC as well as in other cytotoxicity assessments [28]. Recently, a high-throughput bioluminescent cytotoxicity assay based on intracellular protease biomarker release has been developed [29]. ADCC-mediating antibodies were proposed to be a better correlate of protection and prognosis than virus-specific neutralizing antibodies and cytotoxic T cells following anti-HIV immunization and immunotherapy [30]. ADCC activity has been found as an immune correlate that protects against HIV infection and disease progression [31,32]. While weaker protection was observed following influenza A vaccination [33] and in defense against malaria [34]. FcR mediated effector functions including ADCC have also been found to facilitate the clearance of intracellular pathogens including parasitic infections [13,35–37].

2.3. Immunoglobulin class and subclass

Most of the commercial available vaccines mediate humoral protection through induction of serum IgG and mucosal IgG and IgA immunoglobulins (Table 2). Irrespective of the type of vaccine formulation serum IgG levels have been found to correlate with protection against a variety of pathogens. In addition, mucosal IgG levels have also been reported to confer protection [38–41]. IgA is the dominant immunoglobulin in secretions, especially in intestinal and respiratory tracts and mediates protection chiefly through neutralization and less potent opsonization and complement activation than IgG [42–45]. Furthermore, high affinity IgG and IgA antibodies can neutralize bacterial toxins and prevent adherence of pathogenic bacteria to host cells. Mucosal IgG and IgA have been demonstrated as a cocorrelate of protection following intranasal influenza vaccination [46]. It was found during the vaccine trial that both immunoglobulins act synergistically as children negative for both antibodies shed virus 63% of the time compared to just 3% in doubly antibody-positive vaccinated children. Neutralizing antibodies are not always protective but may in some cases be mere indicators of vaccination [47], i.e. non-mechanistic correlates of protection.

2.4. Memory B cells

Memory B cells provide defense in those infections in which the pre-existing antibody levels are very low or if the invading pathogen is able to overcome the existing antibody titers. Memory B cells serve as a second line of immune defense owing to rapid isotype switching, affinity maturation and effector functions. Humoral memory may provide life-long protection against previously encountered pathogens and has been detected more than 50 years after smallpox vaccination [48]. B cell central memory

is prolonged with high level of protective efficacy [49,50]. Functional memory B cells and long lived plasma cells can be measured by ELISpot and more recently by multicolor flow cytometry using markers CD19, CD27, CD45, CD38 and CD138 [51,52]. Memory B cells have been reported to protect against progression to chronic hepatitis following vaccination [53,54]. On the other hand, a very weak correlation was found between circulating intestinal memory B cells in children and protection after vaccination with attenuated human rotavirus vaccine [55]. Similarly, low but significant correlation between numbers of Ag-specific memory B-cells and the corresponding circulating antibody titers were found for measles virus [56] while others have reported no correlation at all [57]. However, therapeutic potential of virus-specific memory B cells have been proven by the demonstration of adoptive transfer of memory B cells [58]. In hepatitis B virus infection, B cell memory acts as a marker of protection and is mediated by the Ag stimulation of memory cells [59].

3. Immune correlates of cell-mediated immunity

It is now well known that for many intracellular infections the development of antibodies is a poor correlate of protection and in recent years a lot of effort has been put into characterization of the more appropriate CMI responses. Several characteristics of T cells such as phenotype, function, Ag-specificity and MHC restriction have been investigated as potential correlates of immune protection in infections and after vaccination (Table 1). Delayed type hypersensitivity (DTH) has commonly been used for the assessment of CMI responses in relation to the infection status and vaccination. However, as the specific antigens are often unknown and DTH via the vaccine preparation will evaluate the entire antigenic repertoire, DTH is a poor immune correlate [60]. Therefore, the immunogenicity against pathogens is better determined by measuring Ag-specific CD4+ and CD8+ T cell responses and their associated cytokines. Recently developed ex vivo assays measure the frequency and phenotype of responding T cells as well as their proliferative potential and killing capacity (Table 3).

3.1. Helper CD4+ T cells and associated cytokines

CD4+ T cells play a central role in coordinating and culminating the adaptive immune response. After recognition of Ag through MHC class II presentation, activated CD4+ T cells differentiate into functional subsets termed T helper 1 ($\text{T}_\text{H}1$), $\text{T}_\text{H}2$, $\text{T}_\text{H}17$, or regulatory T (T_reg) cells (Fig. 1). Effector activity of the $\text{T}_\text{H}1$ cells is brought about by the release of the cytokines such as interferon-gamma ($\text{IFN-}\gamma$), tumor necrosis factor-alpha ($\text{TNF-}\alpha$) and interleukin-2 (IL-2) (commonly referred to as $\text{T}_\text{H}1$ cytokines). In addition, CD4+ T cells provide growth factors and signals for the generation and maintenance of CD8+ T cells [61,62]. Most vaccine studies measure the cytokine secretion by T cells in response to Ag specific for the vaccine as the chief immune marker of CMI induction.

Cytokines can be readily detected at the RNA-level by reverse-transcriptase polymerase chain reaction (RT-PCR) or as proteins in ELISA or ELISpot assays after in vitro stimulation of whole blood or purified cell suspensions with specific Ag [63]. While the ELISA assay provides a quantitative measure of the total cytokine production, the ELISpot assay enumerates the Ag-specific cytokine secreting cells in response to Ag stimulation. Although the amount of secreted cytokines cannot be quantified the ELISpot assay is very sensitive with detection of less than 0.01% Ag-specific T cells. Each of these techniques have been utilized for the development of functional and molecular correlates of vaccine-induced protection [64–69]. At the next level of detail, flow cytometry is a powerful technology that is capable of defining lineage and

Table 3
T cell immunoassays.

Assay	Sample/substrate	Read out	Comments
IFN- γ assay	Whole blood/PBMC	IFN- γ	Cytokine production by all cells in the culture (quantitative)
ELISpot	PBMC	Total T cell response	Number of cells secreting cytokine of interest (qualitative and quantitative), possible to spot two cytokines
Multiplex cytokine assay Cytotoxic assay	Plasma or serum PBMC	Cytokines Cr ⁵¹ , Eu or S ³⁵ release, GAPDH enzyme release, Protease biomarker release	Multiparameter readout T cell-mediated cytotoxicity, ADCC
Fluorescent Ag-Transfected Target Cell (FATT)-CTL assay	PBMC	Lytic activity of virus specific CTL	Ag-specific cell-mediated cytotoxicity
³ H-thymidine proliferation CFSE proliferation	PBMC PBMC	Total T cell response CD4 and CD8T cell proliferation	Single parameter readout Multiparameter readout, functional profile and differentiation
Intracellular cytokine staining (ICS)	Whole blood or PBMC	Surface (CD4 and CD8) and intracellular markers	Multiparameter readout, Ag-specific T cells
Polyfunctional T cells Phosphoflow	Whole blood or PBMC Whole blood or PBMC	Polyfunctional T cells Intracellular signaling molecules (phosphorylation)	Multiparameter readout Multiparameter readout
Tetramer	Whole blood or PBMC	Epitope specific CD8 and CD4	T cell specificity but no functionality, MHC allele typing is required

activation state along with a quantification of cytokine production on a single-cell basis. This is achieved by parallel immune staining of delineation and activation markers expressed on the cell surface as well as secreted cytokines in the intracellular compartment. Labeling of the lymphocyte cell membrane with CFSE prior to cell culture makes it further possible to measure the individual proliferation of cells. CFSE labeling has largely replaced the ³H-thymidine proliferation assay and tracks the cell proliferation by progressive two-fold reduction in fluorescence intensity for each cell division [70]. Technological advancements in multi-parametric flow cytometry now permit the staining and detection of up to 18 different markers, and identified patterns of cytokine secretion can thus be used to differentiate between effector/memory T cell with different immune functions [71]. Intracellular cytokine staining and multi-parametric flow cytometry has been used to suggest immune correlates of protection after vaccination against many human and animal pathogens [72–80].

IFN- γ has been identified as a canonical cytokine of the T_H1 response and plays a crucial role in the vaccine induced protection against several infections [81–83]. One of the best cases for the importance of IFN- γ is the Bacille Calmette Guerin (BCG) vaccine against tuberculosis [84] which generates CD4+ protective T cells producing IFN- γ after stimulation with specific Ag. The detection of Ag-induced de novo production of IFN- γ in cultures of whole blood or peripheral blood mononuclear cells (PBMC) has become a standardized approach for accessing the T cell immune responses following vaccination in chronic infections [83–86]. These IFN- γ release assays (IGRAs) have not only overcome the limitations of DTH but also have improved the ability to distinguish acute and latent infections and in predicting vaccine efficacy [87]. In 2001, QuantiFERON-TB test (QFT) was the first IGRA to be approved by FDA [88]. Cytokine production and T cell proliferation has also been correlated with protection following influenza vaccination in older adults compared to young adults [89,90]. Similarly, T cell mediated protection is needed to control latent infection and disease in cytomegalovirus infection despite the fact that antibodies have been shown to be protective [91,92].

Detection of IFN- γ production does not always reliably predict vaccine success, whether total or individual T cell IFN- γ responses are measured [93]. In some reports protection against *M.*

tuberculosis infection did not correlate with production of IFN- γ by specific CD4+ effector cells [94–97] suggesting the possibility of IFN- γ independent protective mechanisms or the importance of other cellular sources of cytokines. Most recently compelling evidence was provided that CD4+ T cells can induce *M. tuberculosis* growth arrest, even without secreting IFN- γ , TNF- α or both cytokines [98]. While another report showed that Ag-specific CD4+ effector T cell activation is suboptimal in the lungs of tuberculosis infected animals that leads to persistence of infection in the host [99]. These results emphasize that despite the critical role of IFN- γ in T_H1 mediated immunity, this cytokine is not a sufficient immune correlate of protection of its own [100,101].

Another cytokine of effector T cell functional range is TNF- α which mediates killing functions of intracellular bacteria, viruses and parasites [102,103]. With its role in the expansion of T cells, IL-2 has also frequently been associated with the quality of T cell function [104], and these cytokines have been shown to play a role in the host defense against a variety of intracellular pathogens [105–109]. Thus it has been demonstrated that T cells capable of producing multiple cytokines with subsets of IFN- γ , TNF- α and IL-2 correlated well with vaccine induced protection against chronic bacterial and viral infections compared to T cells producing only one cytokine at a time [74,110–114]. These polyfunctional T cells will be described in detail later.

3.1.1. Other cytokines and chemokines

The T_H17/IL-23 pathway can play a crucial role in protective immunity against pathogens [115,116]. It has been shown that ablation of an IL-17 response following both vaccination (Ag-specific production) [115] and infection may lead to reduced protection [116–118]. The T_H17/IL-23 pathway, through $\gamma\delta$ T cells and $\alpha\beta$ CD4⁺ CD8⁺ T cells, is also involved in the induction of neutrophil attracting chemokines and associated protection against pathogens [119,120]. Based on the inputs from mycobacterial infection studies in human and cattle it was proposed that IL-17 may be essential in vaccine-induced control of tuberculosis, keeping in balance other immune responses to avoid immunopathology [121].

A range of cytokines and chemokines have been investigated as potential biomarkers of risk, infection and protection in different infections using techniques such as multiplex bead array,

intracellular cytokine staining and mRNA expression analysis. IL-1, IL-4, IL-10, IL-12, MIP, MIG, IP-10, MCP, TGF- β , GM-CSF have been explored as differentiating biomarkers for infection, vaccines and chemotherapy in serum, plasma, and cell cultures. Additionally, IP-10/CXCL10 produced by monocytes has been recognized as a potential biomarker of latent tuberculosis and following BCG vaccination [122]. MIG and the regulatory cytokines IL-10 and TGF- β 1 correlate with malaria vaccine immunogenicity and efficacy in human volunteers [69]. Beta-chemokines such as MIP-1 α , MIP-1 β , MIP-3 α and RANTES have been found to contribute to protection against infection and in conjunction with vaccination [123–125].

3.2. Cytotoxic CD8+ T cells

In recent years the scientific fields of immunology and biochemistry have seen great advances in the ability to detect and isolate Ag-specific cytotoxic CD8+ T cells (CTL) [126–128]. Successful modifications of methods like the recombinant MHC tetramer approach for staining of specific CTLs [126,129], ELISA [130] and ELISpot [63,131], usage of chimeric molecules and transgenic animals [132,133], and cell proliferation assays to monitor specific CTL killing [134] have all contributed to the increasing amount of data available in regard to cytotoxic immune responses during infection and in vaccination studies. For decades it has been common practice to measure CTL cytotoxicity using the chromium-release assay (^{51}Cr -release). However, a limitation of this assay is the ability to provide only semi quantitative data (unless limiting dilution assays are performed) and furthermore the ^{51}Cr -release assay has a relatively low level of sensitivity [131]. As an alternative, one of the major mechanisms displayed by immune-activated CTLs is the exocytosis of cytotoxic proteins such as pore forming proteins (perforin), serine proteases (granzymes), and cytokines such as IFN- γ , TNF- α and IL-2, IL-4, and IL-10 [135]. Such components, revealing the state of activation for individual CTLs, provide a relatively simple set of cytokines that can be used to monitor and define both CTL frequency and function in responses against infections that require T cells for protection [136]. The IFN- γ ELISpot assay is widely used to monitor specific CTL reactivity against MHC class I binding peptides, to determine specific T cell subsets responsible for a response using receptor-blocking antibodies (Ab), to confirm MHC restriction of specific CD8+ T cell responses, to measure the secretion of IFN- γ triggered by a virus infection and to verify the availability of cross-reactive research tools to expand cytokine studies between species such as cattle and goats [137–139]. A drawback of the ELISpot assay, however, is that the secretion of many cytokines such as IFN- γ is not necessarily a means of CTL activation, but can also be released by non-cytotoxic cells of both the innate and adaptive immune response (Fig. 1). An assay measuring the secretion of molecules associated with lytic activity or the frequency of killing of specific target cells by CTLs would provide another angle to approach the assessment of T cell functional activity. Present and future approaches combining one such assay with highly specific MHC tetramer staining of CTLs and additional flow cytometry-based cytokine release or intracellular staining assays are state-of-the-art in its field and applicable to not only the well known mouse models but almost any species model of interest that being livestock, companion animals or primates. Today such assays are capable of (i) providing a tool for the enumeration and determination of the relative frequencies of epitope-specific, cytokine-secreting CTLs in a sample, (ii) evaluating the effector function of such CTLs, i.e. the identification of specific cytokines and chemokines and their ability to specifically kill infected targets, and (iii) identifying viral as well as tumor derived peptide T cell epitopes [127,134,140].

3.3. MHC tetramers and multimers as immune markers or correlates of protection

MHC tetramers are four recombinant MHC molecules loaded with pathogen-derived synthetic peptides and brought together (usually through the Biotin–Streptavidin interaction) along with a fluorescent tag. They have been well known for more than a decade [126] and are becoming more and more important in the search for new vaccine candidates and as a tool in monitoring immune responses as well as in the enumeration and characterization of T cells [141–143]. With the correct tetramers, Ag-specific CTLs can be directly enumerated *ex vivo* from peripheral blood samples [129,134] although *in vitro* manipulations such as cell enrichment, cell expansion, and/or prolonged stimulation may enhance the individual signals for more detailed analyses [144–146]. However, such *in vitro* manipulations may hinder the accuracy of monitoring the exact responses (frequency and function) of CTLs elicited to disease or vaccination [147]. Despite the established importance of virus-specific CTL-mediated killing of virus infected cells and the possibility to quantify CTLs by tetramer staining of CD8+ T cells, the MHC heterogeneity of any outbred population, and the exclusive specificity of the MHC tetramer with the corresponding T cell receptor, has so far not allowed validation of CD8+ tetramer staining as an immunological correlate of protection. Tetramer stained CTLs are, however, important immune markers of the effects of vaccination as different vaccines or vaccine strategies can be directly evaluated for their induction of peptide-specific CD8+ CTL subsets. Furthermore, tetramers allow the investigation of different T cell engagement patterns with peptide/MHC/ $\beta_2\text{m}$ (pMHC) complexes following vaccination.

3.4. Central memory/effector memory T cells

The goal of an efficient vaccine is to generate long-lived memory CD4+ or CD8+ T cells capable of recognizing and rapidly expanding to combat an infection [148]. Briefly, there are three distinct phases in the generation of memory T cell response: expansion, contraction and differentiation into memory. The expansion phase yields a large population of effector T cells, most of which die in the contraction phase. However, the expansion phase also yields cells that will eventually form the memory cell pool. Immunological memory has been termed as the incomplete unhappening of the primary immune response [149] due to the fact that once the pathogen has been eliminated, most of the activated T cells die. Still a small population survives this contraction phase and become memory cells. Hence, the antigen-activated T cell expansion in a secondary immune response will be more rapid and contraction less severe. Such primed memory cells are maintained for long term after immunization, and cross-sectional studies in humans suggest that the memory response can persist for more than 50 years [150,151]. The memory cell pool is not monolithic. Instead it contains diverse and flexible populations. Two broad categories of memory cells are effector-memory (T_{EM}) (CD62L^{low}CCR7^{low}) and central-memory (T_{CM}) (CD62L^{hi}CCR7^{hi}) cells [152]. T_{EM} cells traffic through nonlymphoid tissues and have high *ex vivo* cytotoxic potential (perforin) but relatively poor proliferative capacity. T_{CM} screen the lymphoid tissues for the presence of specific Ag and have a longer life span and high proliferative capacity to produce effector cells. Very recently an additional memory T cell subset, T memory stem (T_{SCM}) cells, have been reported which exhibits stem cell properties and vigorous proliferative potential [153]. It has been demonstrated in several vaccine systems that long-term protection is often maintained by subsets of memory T cells including T_{EM} cells that rapidly produce cytokine upon re-exposure to Ag along with T_{CM} cells that have the additional property of being able to undergo homeostatic proliferation in order to maintain the memory pool.

T_{CM} and T_{EM} cells may work together to provide optimal immunity against infection [75,114,154]. $CD4^{+}$ and $CD8^{+}$ T_{CM} cells have been correlated with protection and reduced levels of infection in various vaccine experiments primarily dominated by a $TNF-\alpha^{+}IL-2^{+}$ or $IL-2^{+}$ cytokine profile [65,72,77,155,156]. T_{CM} cells have a selective advantage over T_{EM} cells for protective responses against disease owing to higher Ag recall response, high IL-2 production, high killing function in vivo and their location in the peripheral lymphoid organs where Ag presentation and priming occurs. The belief that persistence of the Ag, as after high Ag dose or vaccination, impairs T_{CM} cells and generates memory cells with a T_{EM} profile has been supported by published data [157–159]. These findings suggest a role for T_{EM} cells as correlate of protection following persistence of Ag after vaccination or in cases of high infection. Recently it was found that the detection of multifunctional $CD4T_{EM}$ rather than T_{CM} following parenteral BCG vaccination is due to persisting BCG bacilli post vaccination [160]. $CD4$ and $CD8T_{EM}$ cells that proliferate and produce IFN- γ and $TNF-\alpha$ or IFN- γ alone have been implicated in protection following vaccination against various chronic infections [74,161–164]. Thus the efficacy of T cell-based vaccines could dramatically be improved by manipulating the generation and maintenance of distinct memory T cell subsets. Further the modulation of the composition of existing memory subsets could become a major approach in developing a new generation of therapeutic vaccination.

3.5. Polyfunctional T cells

Recently it has been shown that in several infections the quality of the $CD4^{+}$ (or $CD8^{+}$) T cell cytokine response, as measured through polyfunctional T cells with combined simultaneous production of IFN- γ , $TNF-\alpha$ and IL-2 detected at the single cell level, correlates best with vaccine induced protection [74,79]. Analysis of polyfunctional T cells in response to vaccination should provide a more complete assessment of effector and memory T cells. Furthermore, polyfunctionality of the responses consistently correlate with less pathogenicity and positive disease outcome in multiple disease settings such as *Leishmania major*, HIV-1, HIV-2, hepatitis C virus (HCV), simian immunodeficiency virus (SIV), cytomegalovirus (CMV), *M. tuberculosis* and malaria [72–74,79,165,166]. Many studies have correlated $CD4^{+}$ and $CD8^{+}$ T cell polyfunctionality with vaccine induced long term protective immunity in response to live attenuated [167,168], subunit [72,73,169], plasmid DNA [79,168], recombinant proteins [74,170], peptides [79,165], viral vectored [168] and protein-in-adjuvant vaccines [166,171] as well as prime-boost immunizations [112,172,173]. These studies show a distinct correlation between the frequencies of polyfunctional T cells and the expression of protective immunity and have drastically shifted the focus in T cell vaccine development, evaluation and design towards the capacity of Ag-specific cells to secrete multiple cytokines correlating with protection. Although many reports position polyfunctional T cells in the center of vaccine induced immunity, others have reported that $CD4^{+}$ T cell expression of IFN- γ , co-expression of IFN- γ , $TNF-\alpha$ and IL-2 or $CD8^{+}$ T cell responses did not correlate with risk of disease or protection in cases of tuberculosis and measles infection [97,98,174].

3.6. Innate effectors

Innate effector T cells respond to infection without prior Ag exposure. They are characterized by their location in thymus and a limited T cell receptor (TCR) diversity but rapid effector functions. Invariant natural killer (NK) T cells (iNKT cells) are defined by their expression of a semi-invariant TCR and dependence on either MHC-Ib or CD1d presentation for the Ag recognition. Once activated iNKT cells produce a variety of cytokines (IFN- γ , $TNF-\alpha$,

IL-4, IL-5, IL-13, and IL-17), chemokines and myeloid growth factors and may as such interfere with the interpretation of cytokine production from T cells of the adaptive immune response. The role of iNKT cells has been evaluated in the host response to intracellular infections [175,176]. iNKT cells have limited capacity to expand following antigenic challenge. However, these cells have been found to promote the development of effector and memory T cells when used in conjunction with vaccination [177–179].

The $\gamma\delta$ T cells are another subset of innate T cells defined by the surface expression of the $\gamma\delta$ TCR. They function in immune regulation and epithelial cell repair and play a role in the early response to infections and are primarily located in the mucosal tissues [180,181]. Interestingly, these innate T cells occur in much higher numbers in blood of both cattle and pigs with as many as 10–25% of total circulating T lymphocytes in adult cattle and 12–30% of the total peripheral blood leukocytes in 12 months old pigs. Even higher numbers are observed in young animals with approximately 40% in young calves and 23–57% in 3–4 weeks old pigs [182–184]. It is likely that there are as yet unknown immune functions connected with this abundant T cell phenotype in these species and that they may play an important role in the activation of immune responses and vaccine responsiveness [185,186]. The upregulation of $\gamma\delta$ T cells has also been linked with vaccine induced protection in primates [187] and chickens [188]. Vaccination against chronic pathogens generated potent T_H1 response while the involvement of $\gamma\delta$ T cells induced innate immunity which was associated with blocking the infection [189,190]. Generation of robust $\gamma\delta$ and effector memory T cells was found to be protective against smallpox.

4. Measurements of immune markers and correlates for cell-mediated immunity in large animal species

Similar to medical vaccinology there is an increasing focus on the characterization of CMI responses and the development of protective vaccines against chronic infections in veterinary medicine, although it is a common feature that the inventory of monoclonal antibodies for veterinary species does not match that of mice and men. One of the common assays for a direct CMI read-out is the whole-blood IFN- γ assay followed by quantification through ELISA. Various co-stimulatory cytokines such as IL-2 [191], IL-7 [192], IL-12 [193,194], and IL-18 [191,195] have increasingly found application in such assays for the potentiation of the CMI response. Flow cytometry based evaluation of the T_H1 , T_H2 cells and various cytokines following stimulation with Ag in cultured cells have been successfully demonstrated in various infections in cattle and pigs [78,196–198]. However, the measurement of polyfunctional T cells through multicolor flow cytometry has been severely hampered due to lack of reagents. Recently anti-bovine IL-2 antibodies have been reported along with their use to demonstrate development of polyfunctional $CD4^{+}$ T cells in cattle infected with bovine tuberculosis [199]. In pigs, currently available anti-IL-2 monoclonal antibodies stain a much higher proportion of cells compared to other species, which makes it difficult to perform reliable identification of the rare polyfunctional T cells. In characterization of vaccine induced CMI responses, veterinary vaccinologists have therefore, used IFN- γ mean fluorescence intensity (MFI) as an approximation of polyfunctional T cells as these cells in mice and humans have been shown to make up to 10-fold more IFN- γ on a per-cell basis compared to monofunctional $CD4^{+}$ T cells [200,201]. MFI is a direct correlate of the physical amount of cytokines produced per cell and is a first approximation of the quality of the T cell response [136]. Multiplying MFI with the relative number of cells that make them, i.e. percent positive cells or frequency of Ag-specific T cells, provides a quantitative assessment of the magnitude as well as the

quality of the cytokine response for different cells referred to as the integrated MFI (iMFI) [136]. Most recently, a new index called Generalized iMFI (GiMFI) has been proposed by assigning different weights to magnitude (percentage of positive cells) and quality (MFI) of the responses [202].

New tools for the studies of MHC restricted CD8⁺ responses are still under constant development. Recently, the fully developed large-scale technology to produce, analyze, and validate peptide–MHC (pMHC) interactions which was pioneered over three decades for mice and humans [203–207], has been applied to generation of porcine and bovine MHC molecules. Together with already existing knowledge on T cell epitopes in these species, this has allowed in silico prediction servers that identify specific peptides for MHC binding, such as the artificial neural network based NetMHCpan, also to include cattle and pigs [132,208–212]. Combined with the tetramer technology described above for specific T cell analysis this has enabled fast, accurate “one-pot, mix-and-read” analyses of specific MHC restricted CD8⁺ T cell reactivity following vaccination and/or infection. In pigs, in silico prediction of virus derived peptides bound by swine leukocyte antigen (SLA) class I molecules has been verified by in vitro analysis of specific peptide binding leading to the identification of strongly bound viral peptides, and the effects of T cell specific vaccinations have been investigated by monitoring CD8⁺ T cell responses post vaccination [132,134,212]. For cattle the evidence of a bovine MHC class I (BoLA) restriction of specific CD8⁺ T cells in response to FMDV infection and vaccination has been unveiled by the detection of antigen-specific IFN- γ release from CD8⁺ CTLs in infected and vaccinated cattle of known MHC class I genotypes [137]. Most recently it has been proposed to use immunoinformatics such as the NetMHCpan to predict CTL peptide epitopes in large animals. With expansion of the BoLA repertoire to cover the most commonly expressed alleles, such predictions, followed by experimental verification of T cell specificity to candidate epitopes using pMHC tetramers, will make the prediction and measurement of bovine T cell responses to any pathogen of interest possible [213]. Such reverse immunology proposals were based on recent porcine experiments providing a persuasive proof-of-concept in regard of applying the human MHC project [214] to support the study of livestock immune responses. This was done by producing frequent porcine MHC class I (SLA) molecules, map their peptide-binding motifs followed by the prediction and identification of FMDV derived peptide binders using NetMHCpan leading to the testing of peptide-SLA tetramers on peripheral blood mononuclear cells (PBMCs) from FMDV vaccinated swine [132,134,208,212].

5. Conclusions

In December 2010, global health leaders (WHO, UNICEF, NIH, Bill and Melinda Gates Foundation) called for the next 10 years to be the “decade of vaccines” to research, develop and deliver lifesaving vaccines globally, and especially to the poorest countries [215]. To accomplish this goal it is important to realize that vaccine-mediated immunity is multifarious and cell-mediated and humoral branches do not act in isolation. Rather the best vaccine-mediated protection is achieved when both arms of the adaptive immune system act in concert. Pre-existing antibody titers serves as the first line of adaptive immune defense. However, only when this defense is breached by the infections then recall responses through memory B and T cells come into play. An ideal vaccine should induce residual protective antibody titer, quick effector functions through anamnestic memory B and T responses and active immunomonitoring through rapidly proliferating and differentiating T cells. The immune correlates of protection induced by most current vaccines seem to be

mediated by long-lived humoral immune responses. In contrast, there are no currently available vaccines that are uniformly effective against intracellular infections in which cell-mediated immunity plays a crucial role. The challenge presented in developing potent vaccines against chronic infections, and especially intracellular infections, is unique owing to their complex pathogenesis, limited knowledge about their precise host–pathogen interactions and partially-defined immunological correlates of protection. Considering the dynamic nature of the host–pathogen interaction and display, the establishment of the immune correlates of protection is thus likely to include a combination of both innate and adaptive immune platforms. Thus a significant effort is required to conduct validation and qualification of a proposed correlate before it can become a differentiating immunological correlate of protection through comprehensive evaluation of efficacy and protection. Systems biology offers one integrative approach to analyze the complex immunological networks after vaccination and to predict and comprehensively characterize vaccine response profiling [216,217]. As an example, this approach was used to define the early gene signature (B-cell growth factor TNFRSF17), which predicted the response of B cells, CD8⁺ T cells and neutralizing antibodies following live replicating yellow fever vaccine YF-17D with up to 100% accuracy [218]. Very recently using the same approach to define the early gene signatures, the immunogenicity of human trivalent inactivated influenza vaccine was predicted with up to 90% accuracy [219]. Nevertheless, remarkable progress is being made in our ability to define immunological correlates of protection with the application of novel technologies such as multi-color flow cytometry, high throughput quantitative gene expression analysis, whole genome sequencing, vaccinomics, and reverse immunology and genetics with desired optimism for realization of vaccines for chronic infections.

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ARTICLE IV

A novel multi-stage subunit vaccine against paratuberculosis induces significant immunity and reduces bacterial burden in tissues

Aneesh Thakur¹, Claus Aagaard², Ulla Riber¹, Kirsten Skovgaard¹, Peter Andersen², and Gregers Jungersen^{1*}

¹ *Section for Immunology and Vaccinology, National Veterinary Institute, Technical University of Denmark, Bülowsvej 27, 1870 Frederiksberg C, Denmark*

² *Department of Infectious Disease Immunology, Statens Serum Institute, Artillerivej 5, 2300 Copenhagen, Denmark*

* Corresponding author: Gregers Jungersen

National Veterinary Institute, Technical University of Denmark

Bülowsvej 27, 1870 Frederiksberg C, Denmark

Phone: +45 35 88 62 34

Fax: +45 35 88 66 01

E-mail: GRJU@vet.dtu.dk

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Aneesh Thakur¹, Claus Aagaard², Ulla Riber¹, Kerstin Skovgaard¹, Peter Andersen², and Gregers Jungersen^{1*}

¹ Section for Immunology and Vaccinology, National Veterinary Institute, Technical University of Denmark, Bülowsvej 27, 1870 Frederiksberg C, Denmark

² Department of Infectious Disease Immunology, Statens Serum Institute, Artillerivej 5, 2300 Copenhagen, Denmark

* Corresponding author. Mailing address: National Veterinary Institute, Technical University of Denmark, Bülowsvej 27, 1870 Frederiksberg C, Denmark. Phone: +45 35 88 62 34. Fax: +45 35 88 66 01. E-mail: GRJU@vet.dtu.dk.

ABSTRACT

Effective control of paratuberculosis is hindered by lack of a vaccine preventing infection, transmission and without diagnostic interference with tuberculosis. We have developed a multi-stage subunit vaccine in which a fusion protein of early expressed antigens is combined with a protein expressed in latent infection (FET11 vaccine). FET11 vaccine proteins were formulated with CAF01 adjuvant and injected to MAP challenged calves at two different ages. The FET11 vaccine induced a significant T cell response against constituent vaccine proteins. Of the two different age groups, late FET11 vaccination conferred protective immunity characterized by a significant containment of bacterial burden in gut tissues compared to non-vaccinated animals. There was no cross-reaction with bovine tuberculosis in FET11 vaccinated animals.

Introduction

Mycobacteria are capable of causing deadly infection in cattle populations worldwide with major infections caused by *Mycobacterium bovis* and *Mycobacterium avium* subsp. *paratuberculosis* (MAP). MAP is the causative agent of Johne's disease or paratuberculosis, a chronic progressive granulomatous enteritic disease infecting young calves either via the oral route or in utero infection (41) and characterized by a long asymptomatic period during which the infection is spread. It may eventually cause wasting, weight loss and death months or years after infection due to severe immune pathology and chronic inflammation in the ileum, ileocaecal valve and associated lymph nodes (4, 40). Consequently, substantial economic losses occur at the farm level due to reduced milk yield, premature culling and reduced slaughter value (26). There is additional growing concern about MAP presence in the environment and dairy food products (8) as well as association of MAP with Crohn's disease in humans (25).

Currently available vaccines against paratuberculosis, such as live attenuated or heat-killed MAP, reduce bacterial shedding but fail to prevent transmission or induce sterilizing immunity (14, 29). Moreover, these vaccines results in false-positive reactors on TB skin testing as well as antibody responses in paratuberculosis diagnostic tests. New MAP vaccines including subunit (15, 17), DNA (34), expression library immunization (12), and mutant MAP strains (6) have been tried with partial protection. A number of putative recombinant MAP proteins have been tested in cattle as potential vaccine candidates including heat-shock protein 70 (Hsp70), members of MAP antigen 85 (Ag85) complex, and superoxide dismutase (SOD). Vaccination with MAP Ag85 complex proteins and SOD in MPL adjuvant induced some protection in calves but no significant differences were observed between vaccinated and non-vaccinated groups (15). Also, Hsp70/DDA vaccination has been shown to reduce MAP faecal shedding without differences in tissue colonization compared to non-vaccinated animals (32, 33).

Effective vaccines are lacking for mycobacterial infections including MAP where protection is believed to be dependent on T cell-mediated immunity with CD4+ T cells playing a pivotal role through production of IFN- γ (35). In paratuberculosis, although the infection sets up at an early age, the classical clinical case of disease is an adult animal showing no apparent clinical signs, often recognized as the subclinical shedder (37). Thus, we hypothesize that slow pathogenesis of paratuberculosis can be described as an initial acute, active phase succeeded by a latent, dormant phase in which MAP is hiding inside the macrophages and persists for long periods of time. Further, the protein expression profile changes between the stages with some genes being relatively higher expressed in one or the other stage. We hypothesize that for a vaccine to decrease MAP shedding and prevent reactivation of disease, the vaccine should incorporate different proteins expressed in different phases of MAP infection. Such an approach is called a multi-stage vaccine and has been successfully demonstrated to confer efficient protection in mice models of human tuberculosis compared to BCG vaccine (1). Given the slow pathogenesis of MAP infection, we further hypothesized that the timing of vaccination is more important relative to age where a competent immune response develops, than to precede the time of infection. Here we show that proteins expressed in different stages of MAP infection generate distinct immunological profile and a significant CMI response after vaccination. The purpose of our study was to evaluate the efficacy of a subunit vaccine based on a fusion protein of four MAP proteins from *Esx* family and a latency-associated MAP protein formulated with CAF01 adjuvant. In this study, we show that this approach is quite promising and promotes an efficient immune response that is protective against MAP in experimentally infected calves.

Materials and Methods

Animals

Male jersey calves were obtained over a period of four months from a dairy farm proven to have a true prevalence equal or close to zero by the Danish paratuberculosis surveillance program as reported

earlier (21). A total of 28 calves were acquired with a mean age of 14 days. Animals were housed and raised under appropriate biological containment facilities (BSL-2) located at the institute premises with community pen and straw bedding. This study and all experimental procedures were approved by the Danish National Experiments Inspectorate and all manipulations were performed under their control.

MAP culture and experimental infection

The strain of MAP used for the challenge of the calves was a Danish clinical isolate, Ejlskov 2007, isolated from the faeces of a clinically affected cow in 2007. The strain was growing on LJ medium slants and was propagated on Middlebrook 7H9 medium supplemented with 10% oleic acid-albumin-dextrose complex (Difco, USA) plus 0.05% Tween 80 (Sigma Chemical Co., St. Louis, Mo.) and 2% Mycobactin J (Allied Monitor Inc., USA) at 37°C. Mid-log-phase culture (OD_{600nm}) was centrifuged and counted using pelleted wet weight method that estimates approximately 1×10^7 CFU/mg pelleted wet weight (11). Culture was frozen with 15% glycerin as 1 ml inocula aliquots containing 1×10^9 CFU after validation of purity by performing contamination controls in blood agar plates (37°C, 72 h), Ziehl-Neelsen staining, and IS900 PCR. Two days before inoculation, 1 ml inocula aliquot was thawed in water bath (37°C) and added to pre-warmed media (MB7H9 with supplements; 20 ml) to prepare MAP inoculum for individual animal. The whole suspension was then incubated on a shaker at 37°C for 48 h. MAP inoculation was performed by individually feeding calves with the 20 ml culture suspension in a liter of prewarmed (38°C) commercial milk replacer (DLG, Denmark) thrice within the first week after acquisition (i.e. starting at 2 weeks of age). MAP bacilli in the culture suspension (of the 48 h, 20 ml culture) were enumerated by serial dilution plate counting on Middlebrook 7H10 agar (Becton Dickinson, USA) and cultured for about 4 weeks. Retrospective quantification of CFU's indicated that in total calves received a dose of 1×10^9 live MAP bacilli after three inoculations.

Vaccine groups, antigen, adjuvant and immunizations

All calves were inoculated with MAP. The first four calves were randomly assigned to early FET11, Silirum®, late FET11 and vaccine control groups, respectively. Calves were born over a period of 4 months and followed the same grouping sequence according to date of birth. Calves in early and late FET11 groups received the vaccine proteins twice at the age of 3 and 7 weeks and 16 and 20 weeks, respectively. Silirum® group animals received 1 ml of Silirum® vaccine (CZ Veterinaria, Spain) at the age of 16 weeks as per the manufacturer instructions. Silirum® is a heat-inactivated vaccine containing 2.5 mg of the culture of strain 316F of MAP combined with an adjuvant consisting of highly refined mineral oil. Control calves did not receive any vaccine. The FET11 vaccine antigens, a fusion protein of four MAP proteins ESAP-5, ESAP-2, MAP-3, and ESAP-3 and a MAP latency protein LATP-5 were produced as recombinant antigens. All antigens were produced in *E. coli* and purified by metal affinity and anion columns as previously described (21). Vaccine antigens were allowed to adsorb to the adjuvant CAF01 (DDA/TDB; 2500 µg/500 µg) for 1 h at RT before injection (2). Calves were immunized by the subcutaneous route in the right mid-neck region about 7 cm ahead of the prescapular lymph node. Calves were immunized with 100 µg of each of the fusion protein and LATP-5 in a total volume of 1.2 ml. Blood samples were evaluated for cell-mediated and humoral immune responses and animals were followed for up to 52 weeks (Figure 1).

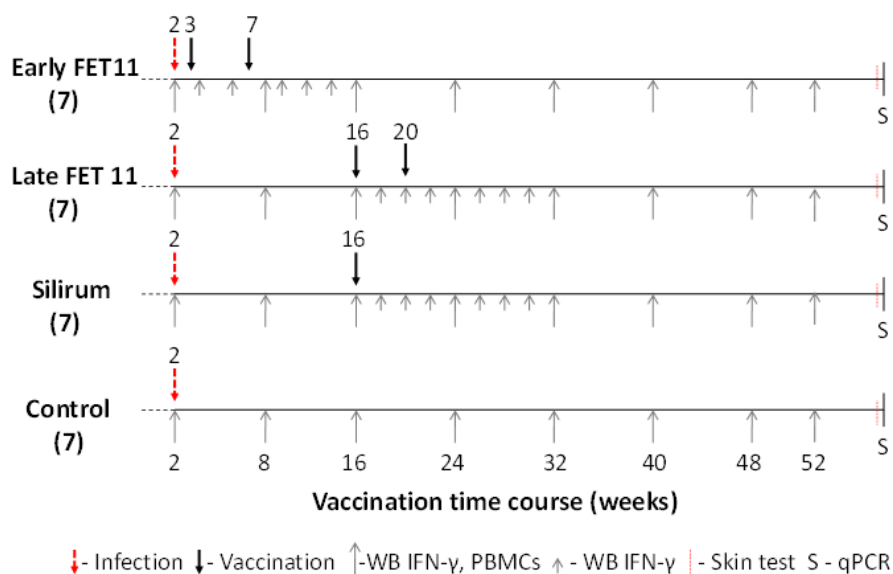


Figure 1. Experimental design.

Whole-blood IFN-γ assay

Whole-blood IFN-γ production was accessed at various time points throughout the study (Figure 1). 0.5 ml heparinized whole-blood was stimulated in 48-well culture plates (Greiner Bio-One, Heidelberg, Germany) with previously added purified protein derivative Johnin (PPDj), purified protein derivative bovine (PPDb) and avian (PPDa) (AHVLA, UK), each of the five MAP vaccine antigens produced individually in *E. coli*, or PBS and two positive control stimulations with phytohaemagglutinin (PHA; Sigma) or the superantigen *Staphylococcus* enterotoxin B (SEB; Sigma), respectively (50 µl volume). All MAP antigens, PPDb, PPDa and SEB were added to a final concentration of 1 µg/ml, while PHA and PPDj were added to a final concentration of 5 µg/ml and 10 µg/ml, respectively. In addition, a non-MAP but TB-specific protein, CFP10 was used at a final concentration of 1 µg/ml in the assay. Whole-blood cultures were incubated for 18-20 h at 37°C and 5% CO₂ in air. Following overnight incubation, 55µl heparin solution (10 IU/ml in blood) was added to avoid clots in the supernatant after freezing. Plates were then centrifuged and approximately 0.35 ml of supernatant was collected into 96-well 1-ml polypropylene storage plates (Greiner Bio-One, Heidelberg, Germany) and frozen at -20°C until analysis.

IFN- γ secretion in supernatants was determined by use of an in-house monoclonal sandwich ELISA as described earlier (22). The levels of IFN- γ (pg/ml) were calculated using linear regression on log-log transformed readings from the two-fold dilution series of a reference SEB stimulated plasma standard with predetermined IFN- γ concentration. The IFN- γ responses in PBS cultures were subtracted from MAP vaccine antigen cultures to generate antigen-specific responses.

Necropsy

The eight first-born calves were euthanized and necropsied at 44 weeks and remainder 20 calves at 52 weeks of age. Six tissue samples from each animal were collected and processed for IS900 qPCR: ileocaecal valve, ileum (0 cm, -25 cm, -50 cm; distance indicated relative to the location of ileocaecal valve in proximal direction), and jejunum (-150 cm, -250 cm). Tissue samples (8 cm in length) were rinsed with sterile PBS. Epithelium, submucosa, and lamina propria were scraped from the serosa with sterile object glass. The tissue scrapings were homogenized by blending in a rotor/stator type tissue homogenizer (Tissue-Tearor, BioSpec Products Inc.) and processed for DNA extraction.

IS900 quantitative Real Time PCR

DNA extraction from tissue samples

DNA was extracted for qPCR analysis from tissue scrapings after bead beating using protocol of the Qiagen DNeasy Blood and Tissue kit (QIAGEN). Briefly, aliquots of 25 mg of tissue scraping homogenates were weighed and incubated on a shaker incubator overnight at 37°C in 400 μ l buffer ATL. Particular attention was given while measuring weight of the tissue homogenates in order to use the precise amount of homogenized material each time. Next day, tubes were subjected to bead beating at full speed for 1 min with 200 μ l of 0.1 mm Zirconia/Silica beads (BioSpec Products Inc.). Bead beaded material were centrifuged for 30 sec at 5000xg, before the 200 μ l supernatants were transferred into new tubes and 20 μ l proteinase-K was added. Tubes were then incubated for 10 min at 56°C. The

mixtures were centrifuged through a Qiagen Spin Column and washed according to the kit protocol. In the final step, DNA was eluted with 200 µl elution buffer and stored at -20°C prior to use.

qPCR assay and standard curves for quantification

Primers for qPCR were acquired (Sigma) referring to MAP IS900-like sequences as has been reported earlier (16). However, we were not able to use these primers because of very low primer efficiency (0.70). Therefore, a new set of primers (F: 5'-GGCAAGACCGACGCCAAAGA-3'; R: 5'-GGGTCCGATCAGCCACCAGA-3') binding to MAP-specific sites of the IS900 were designed using Primer3 (<http://frodo.wi.mit.edu/cgi-bin/primer3/primer3www.cgi>) (31). qPCR reactions were carried out in 25 µl volumes, containing 2.5 µl DNA template, 12.5 µl of 2x QuantiTect SYBR Green PCR Master Mix (Qiagen), and 0.125 µl of 10 µM of each of the forward and reverse primers in nuclease free water. qPCR was performed on a Rotor Gene Q PCR system (QIAGEN). PCR cycling started with an initial denaturation at 95°C for 15 min, followed by 45 cycles of amplification at 95°C for 30 s and 68°C for 60s. After PCR amplification, melting curve data were collected and analyzed.

For quantification of MAP in tissue samples, a standard curve was made by spiking of tissue DNA with DNA from MAP culture (MAP Ejlsov with 1×10^9 CFU/ml) in ten-fold dilutions. DNA from a tissue (jejunum at 250 cm) sample of an animal found to be consistently negative through qPCR was used for spiking. 2 µl of bacterial culture DNA in serial ten-fold dilutions of 1×10^9 organisms was used to spike tissue DNA samples.

Comparative tuberculin skin test

A single intradermal comparative cervical tuberculin test was conducted 3 days before killing the animals according to European regulations (European Communities Commission regulation 141 number 1226/2002) (23). Briefly, 0.1 ml bovine tuberculin (2000 IU) and 0.1 ml avian tuberculin (2000 IU) were

inoculated intradermally in the left side of the neck of each animal. At 72 h post-inoculation, the skin-fold thickness was measured and the increase in the skin-fold thickness compared to day 0 was noted. Reaction to each of the tuberculin was interpreted as follows: a skin test reaction was considered positive when skin thickness increased 4 mm or more, inconclusive when there was an increase of more than 2 mm and less than 4 mm, and negative when the increase was not more than 2 mm.

According to the guideline, the official interpretation of the intradermal comparative cervical tuberculin test is: positive when the positive reaction to bovine PPD is more than 4 mm greater than the reaction at avian site, inconclusive when the positive reaction to bovine PPD is between 1 to 4 mm greater than the avian reaction, and negative when there is negative reaction to bovine PPD or when a positive or inconclusive reaction to bovine PPD is less than or equal to a positive or inconclusive reaction at avian site.

Statistical analysis

MAP vaccine antigen-specific IFN- γ production between different vaccine groups was compared by one-way ANOVA followed by Dunn's multiple comparison test. Data obtained from qPCR (Rotor Gene) was first analyzed through GenEx (MultiD, Göteborg, Sweden) in order to obtain the relative quantities. Based on the dynamic range, a Cq cut-off value of 34 was decided. A Cq value of 34 was assigned to samples with higher Cq or for negative samples. Measured Cq values were then corrected for PCR efficiency to account for suboptimal amplification. Cq values were then converted to linear scale. As the relative quantity values generated from qPCR analysis were not normally distributed, data were converted to base 10 for statistical analysis with parametric methods. Log-transformed data was compared between the four groups for each of the six selected tissue sites from 4 sites in ileum and 2 sites in jejunum. A comparison was also made between the groups on combined ileal and jejunum tissues. At the same time, relative CFU's of MAP were calculated in the tissues from all the animals

based on the standard curve analysis through Rotor Gene software. Protective efficacies of the vaccines were compared by one-way ANOVA followed by Dunn's multiple comparison test using data obtained from both GenEx (\log_{10} RQ) or Rotor Gene (CFU). Log-transformed IFN- γ responses to vaccine proteins or PPDj and relative MAP concentration (\log_{10} RQ) were correlated by a non-parametric Spearman correlation. Statistical analysis was performed using GraphPad Prism software vs. 5.02 (GraphPad Software Inc., La Jolla, CA). $P < 0.05$ was considered statistically significant.

Results

Clinical evaluation

No clinical signs indicative of paratuberculosis were observed among the infected calves. No side effects were observed following subcutaneous FET11 vaccination in calves. However, subcutaneous Silirum® vaccination resulted in a transient nodule of approximately 2-2.5 cm, which subsided in about 3 weeks time.

Immunization with FET11 vaccine induces increasing levels of antigen-specific IFN- γ responses

To examine the immunogenicity and longevity of the FET11 vaccine proteins adjuvanted with CAF01, immune responses were assessed at multiple time points up to 44 (n=8) or 52 (n=20) weeks of age by measuring the vaccine antigen-specific whole-blood IFN- γ responses (Figure 2). In general, control calves did not show antigen-specific response to the vaccine proteins except two animals responding weakly to LATP-5 and ESAP-3, five weeks after MAP infection. In early FET11 vaccinated calves, IFN- γ levels in response to protein ESAP-5 peaked at one week after second vaccination and thereafter remained at low levels throughout the study period. In comparison, late FET11 calves showed immediate increase in levels of IFN- γ after first vaccination that remained consistently higher for a long period but dropped around week 48. Calves vaccinated with Silirum® had consistent ESAP-5-specific IFN- γ production after vaccination with levels in between early and late FET11 groups. In response to ESAP-2, IFN- γ levels for early FET11 vaccinated calves declined 3 weeks after second vaccination and remained at low levels

afterwards. On the other hand, in both late FET11 and Silirum[®] vaccine groups IFN- γ levels peaked after vaccination. However, IFN- γ levels that were higher for late FET11 vaccination than Silirum[®] dropped between weeks 38 up to 48 before coming up again. For the vaccine protein MAP-3, early FET11 vaccinated calves had high IFN- γ levels that waned after week 16 while IFN- γ responses for Silirum[®] calves weakened after week 26.

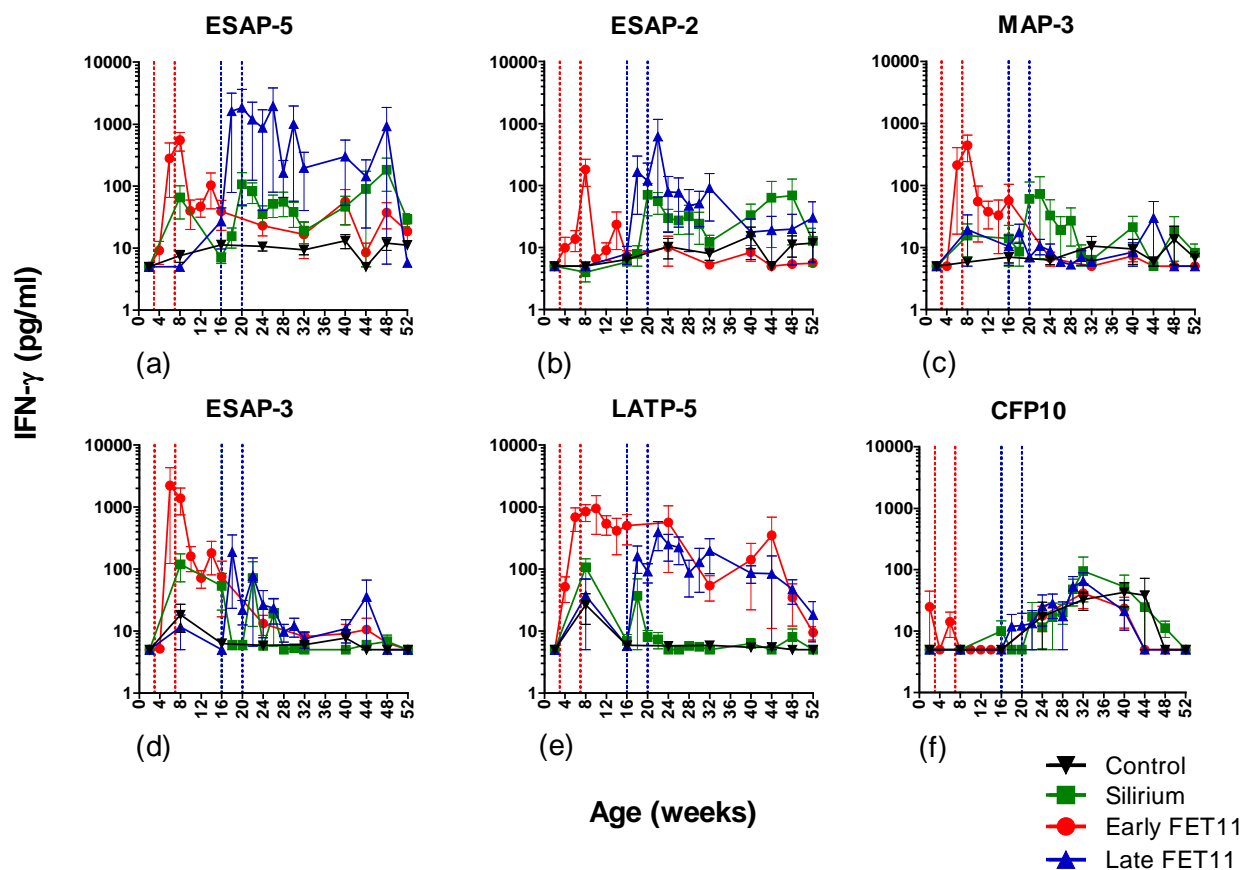


Figure 2. Antigen-specific IFN- γ responses in whole-blood cultures. Levels of IFN- γ released from whole-blood cultures for antigen ESAP-5 (a), ESAP-2 (b), MAP-3 (c), ESAP-3 (d), LATP-5 (e), and CFP10 (f) following vaccinations indicated as dotted lines. IFN- γ levels are expressed as mean values (\pm standard deviation [SD]) for plasma concentrations (pg/ml).

Late FET11 vaccinated calves responded poorly to MAP-3 through all the time points. Production of IFN- γ against ESAP-3 was highest among all groups for early FET vaccinated calves 7 weeks after second vaccination but dropped afterwards. Late FET11 and Silirum[®] vaccinated calves showed low response to this protein. However, Silirum[®] vaccinated calves immediately responded to this protein before Silirum[®] vaccination. Only calves in early and late FET11 vaccination groups responded to LATP-5, with similar response in both groups, starting relative to time of vaccination and dropping around week 46. For the non-MAP TB-specific protein CFP10, responses were found to come up after 20 weeks of MAP infection in all vaccine and control group though the responses were very low. In terms of levels of significance for FET11 vaccine, late FET11 vaccine group responded significantly to ESAP-5 ($p < 0.001$), ESAP-2 ($p < 0.05$) and LATP-5 ($p < 0.05$) as compared to control group. However, early FET11 group had significant responses only to LATP-5 ($p < 0.001$) in comparison with control group. Both early ($p < 0.001$) and late FET11 ($p < 0.05$) vaccine groups had significant differences from Silirum[®] group for LATP-5 induced IFN- γ levels. Silirum[®] vaccine did not have significant differences to control group against any of the vaccine proteins.

qPCR assay performance: dynamic range and specificity

Key performance characteristics of qPCR assay are summarized in table 1. The R^2 value for the spiked tissue standard curve was above 0.99 and the PCR efficiency was 0.97. The detection limit for the spiked tissue was 1.2×10^2 MAP/g tissues. Detection limit was defined as the concentration giving a positive quantification cycle (Cq) value in one or more of the triplicate samples of the standard curves. The lower limits of the dynamic range were based on the mean of the triplicate values and define the quantification limits of qPCR assay. The dynamic range was four log units i.e. $1.2 \times 10^9 - 1.2 \times 10^5$ CFU/g tissue (Figure 3a).

Table 1. Key characteristics of IS900 qPCR assay in spiked tissue samples

Factor	Spiked tissue
PCR efficiency	0.97
Coefficient of Determination (R^2)	0.997
Dynamic range (MAP CFU/g tissue)	$1.2 \times 10^9 - 1.2 \times 10^5$
Detection limit (MAP CFU/g tissue)	1.2×10^2

FET11 vaccination lowers tissue colonization of MAP

At the end of the 44 weeks (n=8) or 52 weeks (n=20) of age, animals were subjected to necropsy. Six tissue samples from the gut of each animal were processed, DNA was extracted and relative quantification of MAP was performed using qPCR. These six tissue sites were selected, as they are more likely to harbor infection based on the prior published knowledge obtained from experimental MAP inoculation studies. Apparently, no significant variation was observed between relative quantities (RQ) of MAP among animals killed at week 44 and week 52. Therefore, data from both the time points were clubbed for analysis when required. In each of the six selected tissues, a lower mean relative concentration of MAP was observed in the late FET11 vaccinated animals as compared to control group (Table 2).

Group	Animal	IC Valve	Ileum 0cm	Ileum -25cm	Ileum -50cm	Jejunum -150cm	Jejunum -250cm
MSV-2 Early FET11	02204	1	1	1	1	1	1
	02214	141	39	36	24	1	1
	02221	28	18	13	26	10	1
	02234	4	1	18	6	1	1
	02250	7203	12390	7203	12390	48	9
	02264	13260	21313	19916	7708	2126	2434
	02291	4	4	16	32	1	1
MSV-2 Late FET11	02201	4	4	26	28	1	1
	02212	260	548	1079	390	39	1
	02219	7	5	9	11	1	1
	02229	1	1	1	1	1	1
	02236	28	26	26	30	13	1
	02269	21	11	7	4	1	1
	02299	880	586	365	243	63	51
MSV-2 Silirum	02197	1	5	1	1	1	1
	02205	16251	34259	29915	17391	3417	4795
	02216	39	13	1	4	1	1
	02225	36	67	30	16	28	12
	02237	36	101	17	20	1	1
	02251	115	198	48	48	9	1
	02279	4481	9447	6731	10819	671	18
MSV-2 Control	02198	1079	1008	769	1514	548	1
	02207	151	243	298	243	162	17
	02217	55	101	11	17	10	6
	02227	151	243	298	243	162	17
	02238	1008	718	298	123	108	123
	02257	55	30	94	67	9	4
	02281	1514	1155	1008	823	173	55

Table 2. Animal-wise relative quantities of MAP in individual gut tissues as quantified by *IS900* qPCR and analyzed through GenEx. Relative quantities of MAP *IS900* qPCR product for ileocaecal valve, ileum 0 cm, ileum -25 cm, ileum -50 cm, jejunum -150 cm, and jejunum -250 cm. Distance in cm indicated relative to the location of ileocaecal valve in proximal direction. Relative quantities were generated from raw Cq values after correction for PCR efficiency using GenEx software.

Silirum® and early FET11 vaccinated animals seemed to have comparable relative MAP tissue load (\log_{10} RQ) in all six tissues with two animals from both groups having very high relative bacterial numbers. Analysis after combining the four ileal tissues and two jejunum tissues revealed exactly similar picture with a mean 1.1 \log_{10} reduction in bacterial load in late FET11 vaccinated animals as compared

to the control group ($p < 0.01$) (Figure 3b and 3c). Analysis of CFU generated through standard curve analysis by Rotor Gene also revealed a mean 1.1 \log_{10} reduction in MAP CFU in late FET11 group compared to control group ($p < 0.05$).

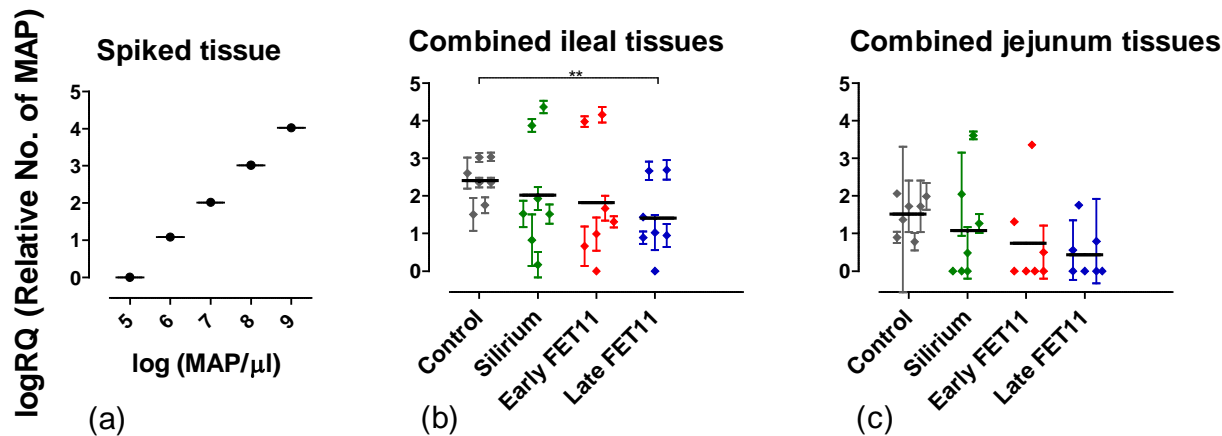


Figure 3. Relative quantities of MAP in combined gut tissues as quantified by *IS900* qPCR. Standard curve of *IS900* qPCR in spiked tissue showing relative quantities (\log_{10} RQ) and \log_{10} MAP CFU (a), relative quantities of MAP (\log_{10} RQ) or *IS900* qPCR product for combined ileal tissues (b), and combined jejunum tissues (c). Relative quantities (\log_{10} RQ) are expressed as mean values (\pm standard deviation [SD]) for relative number of MAP (b and c).

PPDj-specific IFN- γ production correlates with MAP load in tissues

All animals exhibited progressive PPDj-specific IFN- γ production following MAP infection (Figure 4a). Characteristically, in early FET11 vaccine group these responses declined after second vaccination but came up again after 8 weeks. Towards the advanced stages, early FET11 vaccinated animals had lower PPDj-specific responses than Silirum® or control group. Late FET11 vaccinated animals had consistently lower PPDj-specific responses after vaccination. In comparison, Silirum® and control group animals showed an increasing trend of IFN- γ responses against PPDj towards the advanced stages of the study (Figure 4b). A positive linear correlation between the log-transformed PPDj mean IFN- γ responses and

relative quantities of MAP (\log_{10} RQ) was found at weeks 48 ($p < 0.05$) and 52 ($p < 0.01$) post infection. These results were supplemented by the observation that an inverse statistical correlation was found between the relative quantities of MAP (\log_{10} RQ) and log-transformed mean IFN- γ responses against vaccine proteins at weeks 24, 32 and 40 of the study ($p < 0.05$). IFN- γ responses to PPDj also correlated with MAP CFU calculated through standard curve at weeks 32 ($p < 0.001$), 40 ($p < 0.05$) and 52 ($p < 0.05$) of the study. In terms of vaccine group, we could see a significant correlation between IFN- γ responses to pooled vaccine proteins and MAP load in tissues for late FET11 vaccine group only at week 32 ($p < 0.05$). There was a correlation between IFN- γ response to vaccine proteins, ESAP-2, ESAP-5 and reduced bacterial load at week 22, 24 and 32 ($p < 0.05$).

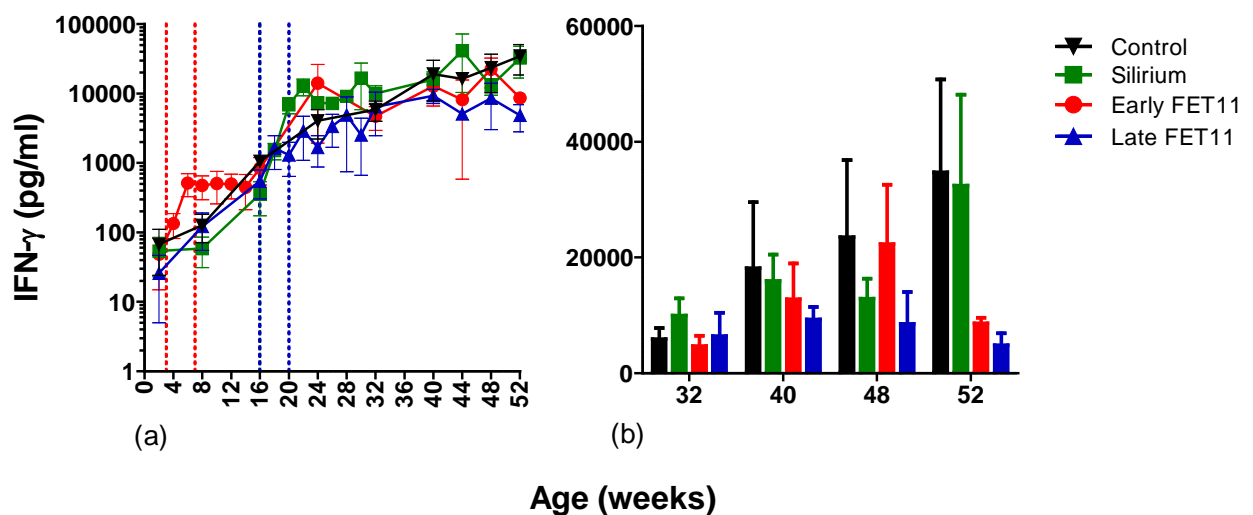


Figure 4. PPDj-specific IFN- γ responses in whole-blood cultures. Levels of IFN- γ released from whole-blood cultures stimulated with PPDj relative to MAP inoculation (a) and between 32-52 weeks of age (b). IFN- γ levels are expressed as mean values (\pm standard deviation [SD]) for plasma concentrations (pg/ml).

Tuberculin skin testing

All the animals in the experiment were subjected to comparative intradermal tuberculin testing 72hrs before killing at the end of the week 44 ($n=8$) or 52 ($n=20$) (Figure 5). In Silirum® group, four animals had

positive skin reactions to PPD_b while majority of animals from other groups had either negative or inconclusive reactions to PPD_b. Most animals from the four groups had positive reactions to PPD_a with strongest responses among the Silirum[®] vaccinated group. One animal from the Silirum[®] group had a positive comparative tuberculin test. However, majority of the animals from the four groups had reactors to PPD_a. Only one animal each from control and Silirum[®] groups had a positive reaction to PPD_b.

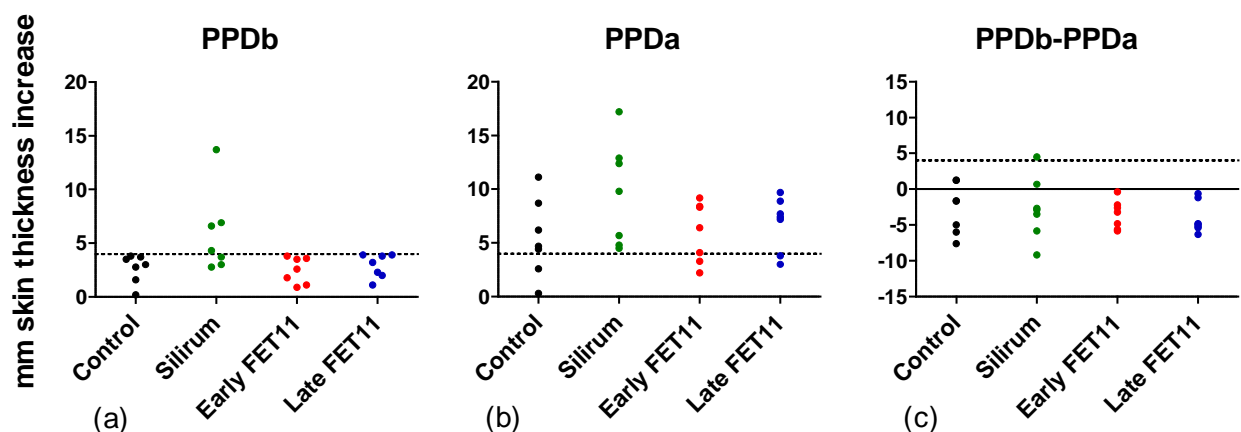


Figure 5. Increase in skin thickness after the comparative cervical intradermal tuberculin test three days before killing. Results are expressed as mm skin thickness increase for PPD_b (a), PPD_a (b), and mm size difference PPD_b-PPD_a (c) over a 72 hr period. A threshold of 4 mm over which an animal has a positive reaction, is shown by a horizontal dotted line. Skin thickness (mm) is depicted for all individual animals grouped by treatment.

Discussion

We have described a post exposure vaccine approach conceived to incorporate a single fusion protein of four early MAP proteins and a latency-associated protein as a novel subunit multi-stage vaccine, FET11. We hypothesize that such a vaccine could decrease MAP shedding and prevent reactivation of disease. Similar approach has demonstrated an efficient protection in mice models of human tuberculosis as compared to BCG vaccine (1). Five MAP proteins were selected based on previous immunogenicity

studies in cattle and experimental knowledge from *in vitro* and *in vivo* expression studies with *M. tuberculosis* proteins in mice. (20, 21). Results show that vaccination with the FET11 vaccine after experimental infection of MAP results in significant CMI responses and a mean 1.1 log₁₀ reduction in bacterial colonization of the gut in comparison with non-vaccinated animals at 8-12 months post MAP inoculation. Using the similar vaccine strategy, older animals developed a more robust immune response and were better able to check MAP load in the tissues even when the infection occurs at an earlier age. A discrete immunological profile for the five constituent FET11 vaccine proteins among early and late vaccinated animals was observed. Proteins of *Esx* family, ESAP-2 and ESAP-5 and a latency protein, LAMP-5 were found to be highly immunogenic. Notably, animals vaccinated with FET11 vaccine at an older were better able to contain MAP infection in gut tissues as compared to a commercial whole-cell heat inactivated vaccine Silirum® or early FET11 vaccinated animals. Nevertheless, immune response to FET11 vaccine proteins was evident in Silirum® vaccinated animals but was characteristically absent against proteins ESAP-3 and LAMP-5. FET11 vaccination did not lead to a significant induction of false positive reactors in the intradermal tuberculin test for bovine tuberculosis vis-à-vis Silirum® vaccine. In addition, an experimental challenge model in calves using mid-log-phase frozen stock MAP cultures and the correlation of infection with PPDj responses has been described. This emphasizes the possibility of establishing a uniform and reproducible bovine MAP infection model despite non-availability of age matched calves in desired numbers at the same time for vaccine experiments.

Significant differences in the vaccine-induced CMI responses to component proteins were observed between late FET11 vaccine group and control group over a long period after experimental challenge. These vaccine-induced IFN-γ responses were antigen-specific as indicated by the fact that control animals did not show any antigen-specific immune response following challenge. However, majority of the animals responded to a non-MAP TB-specific CFP10 protein between 4-7 months of age. Although levels were very low, we could not explain this considering the design of our experimental setup where

the animals attained this age over a four month period. There has been compelling evidence that T_H1 type immune responses, chiefly IFN- γ , is essential for immunity against mycobacterial infections including MAP (9, 36). In late FET11 group, immunogenicity and longevity of vaccine-induced immune responses were most stable for ESAP-2, ESAP-5, and LATP-5. But we did not find a strong correlation between IFN- γ productions to every component vaccine protein and reduced bacterial burden in the gut tissues after vaccination. However, we found that the vaccine protein pool was significantly correlated with reduced bacterial number at week 32 for late FET11 group. Interestingly, LATP-5 induced IFN- γ production was characteristically absent from Silirum[®] and control group. Since we did not have a non-infected vaccine group, we do not know how the infection influences the observed IFN- γ responses to the vaccine. ESAP-2 and MAP-3 recombinant proteins were found to be low immunogenic in this study. This contrasts our earlier report in which significant IFN- γ responses were observed against both of these proteins after vaccination (Article 1). We do not understand this discrepancy but it emphasizes the possibility of dynamics of MAP infection in guiding the differential immune response to component vaccine proteins.

We have previously shown that immunological maturity of the animals does seem to play an important role in vaccine-induced immune response (Article 1). This study also highlights the fact that despite getting exposed to the bacteria at the same time, age of vaccination and regimen do play a role in vaccine-induced immune responses and subsequent containment of infection as evidenced by significantly reduced bacterial load in FET11 animals vaccinated at an older age. Young calves in first 6-8 months of life have relatively higher number of $\gamma\delta$ T cells and NK Cells compared to adult cattle (18). The role of $\gamma\delta$ T cells (28), NK cells (24) and polyfunctional T cells (19, 39) have been appreciated in mycobacterial infections but their precise contribution in MAP infection is yet to be fully elucidated.

427 Quantitative real time PCR (qPCR) is a rapid and sensitive method for quantification of target DNA. Due
428 to slow growth, long generation time and tendency of MAP to form aggregates, qPCR has been
429 increasingly used for paratuberculosis diagnosis as compared to culturing (16, 27). The *IS900* element is
430 an insertion sequence considered to be a MAP-specific gene (10). *IS900* qPCR is a highly sensitive and
431 specific method for the detection of MAP due to multiplicity of *IS900* within MAP genome, with one
432 genome of MAP containing 15-20 copies of *IS900* gene (10). In addition to diagnostics, qPCR has been
433 used as a tool for monitoring of microbial load in tissues, disease pathogenesis, and efficacy of vaccines
434 and drugs (7, 30). In our study, we exploited the potential benefits of qPCR based on *IS900* gene to
435 monitor the MAP load in the tissues samples and did a relative quantification of bacterial numbers in
436 vaccinated versus control animals. The qPCR assay was validated based on a previously published report
437 (16). However, we designed a new set of primers specific for MAP *IS900* avoiding *IS900*-like sequences
438 and having a relatively close T_m compared to ones in the published report. With these new primers we
439 found a higher PCR efficiency. Determination of parameters such as PCR efficiency, dynamic range, and
440 detection limit are important for qPCR assay (5). Our qPCR assay displayed optimal reaction conditions
441 as evidenced by high efficiency of standard curve for spiked tissue sample (Table 1). Cq values obtained
442 with qPCR assay were analyzed with GenEx® software. GenEx is intuitive software involving sequential
443 analysis of data such as efficiency correction, calibration, normalization, relative quantification etc. and
444 offers distinct advantages over $\Delta\Delta Cq$ method. The relative CFU calculation by standard curve method
445 supplemented the results from GenEx analysis and emphasizes the usefulness of both the approaches.
446
447 Vaccination against paratuberculosis interferes with diagnosis of tuberculosis on skin testing. In this
448 study, the response of FET11 vaccinated animals to PPDb was lower than PPDa after comparative
449 tuberculin skin testing. There were one reactor each from control and Silirum® vaccine groups and a
450 positive animal among Silirum® vaccinated animals. Thus, an inference can be drawn that FET11

vaccination may not interfere with the official diagnostic test for tuberculosis when comparative skin test is used.

We found a positive correlation between whole-blood IFN- γ responses against PPDj and relative number of MAP in gut, which was especially significant in advanced weeks of the experiment. PPDj is a crude antigenic mixture of a number of proteins. The use of PPDj for CMI based diagnosis of MAP has been challenged because of low specificity due to cross-reactive antigens (13). However, PPDj responses could be a good measure of tracking infection status in experimental MAP infections and thus can serve as an immunological correlate of infection for vaccine experiments. At the same time, the lack of a strong correlation between reduced bacterial burden and whole-blood IFN- γ responses to component FET11 vaccine proteins in our study could challenge the key role of IFN- γ as an immunological correlate of protection in MAP. Else, antibodies or polyfunctional T cells may play an important role in vaccine-induced protection (17) that needs further verification.

A number of recombinant MAP proteins have been tried and tested as subunit vaccines in calves. Study using proteins of antigen 85 complex and superoxide dismutase (SOD) with MPL or recombinant IL-12 DNA as adjuvant reported some protection but did not find significant differences between any vaccinated groups (15). Recombinant MAP Hsp70/DDA vaccination was found to reduce bacterial shedding during almost two years after challenge in calves possibly through induction of antibodies (17). However, recent report suggest that this vaccine was unable to lower tissue colonization and initial faecal shedding of MAP (33). Moreover, Hsp70 vaccination did not induce strong antigen-specific IFN- γ responses compared to control animals. Contrary, we found significant antigen-specific IFN- γ responses in our study. Though, there was a differential immune response to constituent proteins, the delivery of selected candidates as a single fusion protein has the potential benefit of enhanced responses to low responder proteins such as MAP-3 and ESAP-3 in our study. Similar observation was found for low

immunogenic ESAT-6 when used with antigen 85B in a strong adjuvant DDA-MPL (3, 38). At present, levels of MAP shedding in faeces and the development of an antibody response to MAP and vaccine antigens in our study remains to be analyzed.

Summarized, our study demonstrates that a multi-stage subunit vaccine based on a fusion protein of early secreted MAP proteins and a latency-associated MAP protein formulated in CAF01 adjuvant is able to reduce bacterial colonization of gut in challenged calves. Considering the slow pathogenesis and prolonged subclinical phase of MAP infection in cattle, further studies are required to evaluate protective efficacy and immunological memory of FET11 vaccine over a longer period of time. The possible role of various T cells and innate immune cells as an immune correlate responsible for the observed protection need to be further investigated. Our results lay the groundwork for the evaluation of possible long-term preventive and therapeutic effect of FET11 vaccine in chronic naturally infected cattle.

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PhD Thesis
Aneesh Thakur

National Veterinary Institute
Technical University of Denmark
Bülowsvej 27
1870 Frederiksberg C

Tel +45 35 88 60 00
Fax +45 35 88 60 01
www.vet.dtu.dk

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